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Increase in the Process of Accumulation of Amino Acids in Carrot Slices with Prolonged Aerobic Washing

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The ability of slices of storage tissue of certain plants to accumulate inorganic ions (Asprey, 1936–37; Stiles & Dent, 1946; Rees, 1949; Sutcliffe, 1952) and amino acids (Birt & Hird, 1956, 1958*a*) is markedly stimulated by a prior period of aerobic circulation in water. Related phenomena are the development of salt-stimulated respiration (Robertson, 1941) and malonate-sensitivity of respiration (Hanly, Rowan & Turner, 1952; Laties, 1959) on aerobic washing of plant storage tissue. The factors underlying the development of these apparently changed physiological states are unknown.

The results reported here are an attempt to assess the importance of some of the processes occurring during the washing procedure. They indicate that during aerobic circulation of the tissue, removal of competitors (endogenous material) for the mechanism of accumulation is associated with increased accumulation of amino acids and salt-stimulated respiration.

MATERIALS AND METHODS

Materials

The amino acids and sulphanilamide used were commercial samples. Extracts and solutions of amino acids were adjusted to pH 5·0 with HCl before use. Commercial oxygen-free nitrogen was used as the means of circulating carrot slices anaerobically.

Estimation of amino acids

Amino acids were measured by direct development of the colour in samples of the solution, with 2 ml. of the ninhydrin reagent of Connell, Dixon & Hanes (1955). The samples (40 μl.) were pipetted from a semi-automatic pipette on to pieces of Whatman no. 3 paper (1 cm.^{2}) prepared by the method of Connell *et al.* (1955). This method was also used to determine the concentration of ninhydrin-reacting material (amino acids plus ammonia) in the carrot extracts obtained, and in assessing leakage from the carrot tissue into the external medium.

Histidine was estimated by the method of Macpherson (1946) with the modifications of Birt & Hird (1956).

Tissue extracts

Ethanolic extraction. Carrots were peeled and cut into slices 1 mm. thick; 500 g. of slices was added to 2 l. of ethanol (80%, v/v). The extraction proceeded, with occasional shaking, for 16 hr. at room temperature. The extract was then poured off and replaced by fresh 80% ethanol (1 l.) and this was allowed to stand, with frequent shaking, for a further 2 hr. The total extract (3 l.) was then filtered to remove suspended material and concentrated under reduced pressure at 35°. In order to remove the last traces of ethanol, the residue was taken up in 100 ml. of water which was dried under reduced pressure. The process was repeated with 50 ml. of water. The residue was dissolved in water to give the final extract, which was adjusted to pH 5·0 and made up to give a final concentration of 50 mM with respect to ninhydrin-reacting material. Undissolved material was removed by centrifuging. The concentrated extract was analysed for material reacting as histidine (histidine, tyrosine and phenols) by the method of Macpherson and found to be 0·46 mM with respect to a histidine standard. The final volume was 150 ml.

Boiling water extraction. Slices (500 g.) (1 mm.) were added, over a period of 2 min., to 3 l. of boiling water and boiled for 5 min. The container was then rapidly cooled with water and allowed to stand for 20 hr. The extract was then filtered and concentrated under reduced pressure as before and made up to give a final concentration of 50 mM with respect to ninhydrin-reacting material. The concentrated water extract was analysed for histidine-reacting material and found to give a colour yield equivalent to 0·37 mM-histidine. The final volume was 152 ml.

Both extracts were treated with 1 g. of activated carbon/100 ml., being briefly brought to 60° as the carbon was added, and cooled rapidly immediately after the addition. This treatment decreased the turbidity of both extracts and diminished the orange colour of the ethanolic extract.

Tissue

Carrots were obtained not more than 24 hr. after harvest, and sliced on the day they were obtained. Slices (1 mm.) were prepared by the method given by Birt & Hird (1956) and were taken from the cambial zone. For uptake of amino acids the slices were cut into pieces (20–60 mm.^{2}). In order to obtain greater reproducibility in sampling for respiration experiments, disks (0·4 cm. in diameter) rich in cambial tissue were prepared from the slices with the aid of a stainless-steel cork borer.

The freshly cut slices or disks were given several preliminary washings to remove cell and surface debris and to mix the pieces of tissue. In experiments where some tissue was to be circulated aerobically and some anaerobically, the pieces of tissue were always mixed before being divided.

Circulation was carried out in 1 l. round-bottomed flasks through which air was drawn and which were kept at 25° in a constant-temperature room. Sulphanilamide (mM) was

present in the circulating water to inhibit the growth of bacteria. A previous experiment had shown that the tissue exhibited salt-stimulated respiration and active uptake of amino acids after circulation under these conditions. When simultaneous circulation under anaerobic and aerobic conditions was used, gas flows of air and nitrogen were regulated until the rates of circulation of the pieces of tissue were approximately equal. After circulation for 0·5–1 hr. the fluid was replaced several times with water, and finally water containing sulphanilamide was added. This procedure removed substrates for bacterial multiplication, which are released by the tissue after it is cut. After this initial replacement, the circulating medium (water containing sulphanilamide) was changed every 24 hr. Sulphanilamide was not used in the final incubation medium except in one experiment, which was of 24 hr. duration. Here mM-sulphanilamide was also used.

Before being used in an experiment the pieces of tissue were washed in water several times and blotted lightly with hardened filter paper.

Experimental procedure

Measurement of oxygen consumption. A circular Warburg apparatus was used. About 40 disks were placed in 2 ml. of incubation fluid in the main compartment. The total 'fluid volume' in the main compartment was determined by taking the specific volume of the carrot disks to be 1 and making a correction for the volume obtained from the fresh weights of the disks (approx. 0·4 ml.). The centre well contained NaOH. The bath temperature was 25° and the manometers were shaken at 160 oscillations/min. The gas phase was air.

The tissue was allowed to pre-incubate for 15 min. before the first reading. Experiments generally lasted 2–2·5 hr., at the end of which the disks were removed, blotted lightly and weighed. Dry weights were determined after drying overnight at 105°.

Measurement of amino acid uptake. Uptake of amino acids by the tissue was followed by determination of the change in concentration of ninhydrin-reacting material in the medium. Tissue during the experimental period did not exhibit a net uptake or release of water.

The tissue slices (about 2 g.) were weighed and placed in 10 ml. of the incubating solution at pH 5·0 in a stoppered conical flask (50 ml.) and shaken at 150 oscillations/min. in a water bath at 25°. The stopper was removed twice for a few minutes during the experiment to allow gas exchange. Samples were removed for analysis at appropriate intervals.

Chromatography of boiling-water extract. Descending paper chromatography was used with Whatman no. 1 paper. The moving phase was butanol–acetic acid–water (4:1:1, by vol.) and also phenol saturated with water.

RESULTS

Effect of alternate aerobic and anaerobic circulation on salt-stimulated and control respiration. Fig. 1 shows salt-stimulated respiration expressed as a percentage of control respiration. It is seen that in unwashed tissue potassium chloride (50 mM) slightly depresses respiration. After 64 hr. of aerobic circulation, the presence of potassium chloride stimulates respiration by 43%; these results are in keeping with earlier work (see Robertson, 1941). Tissue having developed this salt-stimulated respiration is shown to lose it after a further 70 hr. circulation under anaerobic conditions, and its respiration is actually inhibited in the presence of potassium chloride. On being re-circulated under aerobic conditions for a further 68 hr. the salt-stimulated respiration is restored and increases to 82% above the control respiration.

Tissue from the same batch, when initially circulated anaerobically, does not show salt-stimulation. With changes from aerobic to anaerobic circulation, there is the same stimulation and inhibition of respiration by potassium chloride as occurs in tissue initially circulated aerobically.

Experiments were not continued beyond 200 hr., which was in the region of the life of the tissue under the conditions used. The results are expressed as percentages of controls receiving similar aerobic and anaerobic treatment. The control respiration generally increased after aerobic circulation and decreased after anaerobic circulation.

Increased uptake for some D- *and* L-*amino acids after circulation.* Birt & Hird (1958*a*) showed that isoleucine and valine were rapidly accumulated by tissue after circulation and that this accumulation was not complicated by metabolism of these amino acids. They were therefore chosen here for experiments designed to follow the development of the uptake process. Further it had been shown in the above-mentioned paper that the L-isomers of amino acids had a greater affinity for the uptake process than the D-isomers.

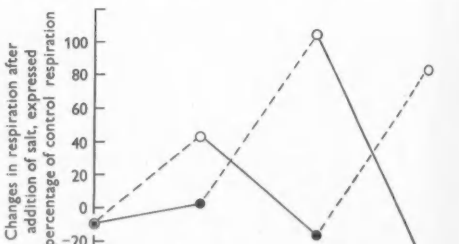

Fig. 1. Effect of alternate aerobic and anaerobic circulation on salt-stimulation of respiration. The tissue (0·4 g.) was in 2 ml. of water or 2 ml. of 50 mM-potassium chloride. Incubation was carried out at 25° for 2 hr. Experiments were performed in triplicate and means were plotted. - - - ○, Period of aerobic circulation; —●, period of anaerobic circulation. Change in respiration is expressed as percentage of control respiration on a fresh-weight basis. Maximum deviation of Q_{O_2} values from mean is 13%.

Birt & Hird (1956, 1958*b*) showed that 0·1 mM-2:4-dinitrophenol markedly inhibited the uptake of amino acids. Although some work has been done on the dinitrophenol-insensitive uptake it was felt that dinitrophenol-sensitive uptake, being considerably greater, would be a useful fraction of active uptake for study.

Fig. 2 shows that in tissue freshly cut there was substantial dinitrophenol-sensitive uptake of L-isoleucine but considerably less of the D-isomer. Further it is shown that the percentage increase after a period of aerobic circulation was larger with the D-isomer than with the L-isomer. The graph shows that after 21 hr. circulation under aerobic conditions, no further increase in dinitrophenol-sensitive uptake for either D- or L-isoleucine occurs. Additional experiments showed that occasionally there was a small increase after 21 hr. Similar results were obtained with D- and L-valine. About 60 hr. was used as the circulating period in subsequent experiments, as this period represented termination of the development of uptake.

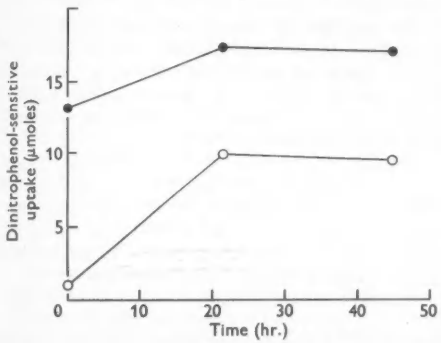

Fig. 2. Effect of aerobic circulation on development of accumulation of D- and L-isoleucine: ○, D-isoleucine; ●, L-isoleucine. Tissue (2 g.) was incubated in 10 ml. of appropriate solution (5 mM) with and without dinitrophenol (0·1 mM). The initial pH was 5·0 and duration of uptake 6 hr.

Table 1. *Effect of washing on dinitrophenol-insensitive uptake of* D- *and* L-*isoleucine*

Tissue (2 g. fresh weight) was incubated for 6 hr. in 10 ml. of solution at 25°. Initially, the pH was 5·0, the concentration of D- and L-isoleucine was 5·0 mM and of dinitrophenol 0·1 mM. The uptake period was 0–6 hr.

Time of washing (hr.)	Uptake (μmoles)	
	D-Isoleucine	L-Isoleucine
1	0·8	0·6
22	2·1	4·5
45	2·3	5·1
65	7·6	12·5

Table 1 shows that dinitrophenol-insensitive uptake of both D- and L-isoleucine also increases during circulation. After circulation dinitrophenol-insensitive uptake of L-isoleucine is at all times greater than the similar uptake of D-isoleucine. Similar results were also obtained with D- and L-valine and suggest the operation of a carrier mechanism with an energy requirement insensitive to dinitrophenol (? glycolysis). The results again show the increase in the process of uptake although with this minor component there is no evidence for the differential increase of uptake of the D-isomer.

Dinitrophenol-sensitive uptake of some D- *and* L-*amino acids after alternate aerobic and anaerobic circulation.* With the D- and L-isomers of valine and isoleucine, an increase in dinitrophenol-sensitive uptake during the first 45 hr. of circulation always occurred under aerobic conditions. This increase was considerably greater with the D-isomer than with the L-isomer. During initial anaerobic circulation there was often a fall in dinitrophenol-sensitive uptake, but after the first circulation period a marked and uniform response has been repeatedly obtained, i.e. dinitrophenol-sensitive uptake increased after aerobic circulation and decreased after anaerobic circulation. Results obtained with D-isoleucine are shown in Fig. 3.

A similar result was obtained with L-isoleucine, L-valine and D-valine. Once again changes in active uptake were always less marked for the L-isomers than for the D-isomers.

Salt-stimulated respiration and amino acid uptake from the same batch of tissue. With the same batch of tissue, the effect of alternate aerobic and anaerobic circulation on unstimulated respiration, salt-stimulated respiration Q_{O_2} of the tissue and dinitrophenol-sensitive uptake of D-isoleucine was tested simultaneously. These all increased after aerobic circulation and all decreased after anaerobic circulation, as in previous experiments in which batches of different tissue were used. The results obtained

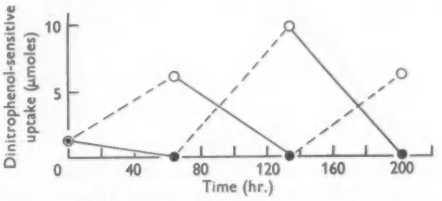

Fig. 3. Effect of alternate aerobic and anaerobic circulation on dinitrophenol-sensitive uptake of D-isoleucine. The tissue (2 g.) was in 10 ml. of D-isoleucine (5 mM) and 10 ml. of D-isoleucine plus dinitrophenol (0·1 mM). Initial pH was 5·0 and duration of uptake 6 hr. - - -○, Period of aerobic circulation; —●, period of anaerobic circulation.

therefore describe a common behaviour of carrot tissue under the conditions used.

Ninhydrin-reacting material in the tissue. It had been observed that ninhydrin-reacting material is released from freshly cut carrot tissue, and in order to obtain more quantitative information about this material the amount extractable by 80% ethanol has been determined. The results for eight carrots from the same batch indicate that surprisingly high concentrations (35–61 mM) of ninhydrin-reacting material are found in the tissue.

These results were felt to be of interest in relation to the observed competition for the carrier system between externally supplied amino acids (Birt & Hird, 1958*b*). It was decided therefore to investigate the process of leakage of ninhydrin-reacting material from the tissue, as it was thought that migration of endogenous amino acids during the circulation process might be related to the development of active uptake.

Release of ninhydrin-reacting material during initial aerobic circulation. Release from freshly cut tissue into water, and water containing 0·1 mM-dinitrophenol, was studied. The particular tissue had been determined on the basis of its water content to be 50 mM with respect to ninhydrin-reacting material. Results over 6 hr. are shown in Fig. 4. In the presence of water and water containing dinitrophenol, 3 and 8% respectively of the endogenous amino acids were lost after 6 hr. In water a maximum concentration was obtained at 3 hr. incubation, i.e. after 3 hr. the rate of reabsorption exceeded the rate of release. In another experiment reabsorption had removed almost all the amino acids from solution after 12 hr. In the presence of dinitrophenol the rate of leakage exceeds the rate of any reabsorption which may occur.

Release of ninhydrin-reacting material after prolonged aerobic and anaerobic circulation. Release into water was extremely small after prolonged circulation whether the tissue had received either aerobic or anaerobic treatment.

Release into 0·1 mM-dinitrophenol was much greater, and showed marked fluctuations with change of circulation procedure. After the initial aerobic or anaerobic circulation period of 60 hr., release into dinitrophenol is variable but comparatively low (Fig. 5). After this period prior anaerobic circulation causes a marked stimulation of release, whereas prior aerobic circulation either causes no change or reverses the effect of anaerobic circulation.

The processes of leakage of ninhydrin-reacting material into dinitrophenol solution after aerobic and anaerobic circulation thus show similar responses, but opposite in sign, as do salt-stimulated respiration and amino uptake under the same conditions.

The reversal of the process of leakage, the development of salt-stimulated respiration and active uptake of amino acids are thus associated with aerobic circulation; anaerobic circulation reverses these processes. These effects are likely to have a common basis.

Effect of cold-water extract on uptake of L-*histidine.* It was decided to determine whether material released into cold water by freshly cut tissue was responsible for the inhibition of uptake of amino acids in unwashed tissue. Because of its relatively specific method of assay, L-histidine was chosen for this aspect of the investigation. A cold-water extract (3 hr.) was prepared and its effect on the uptake of L-histidine was tested with aerobically

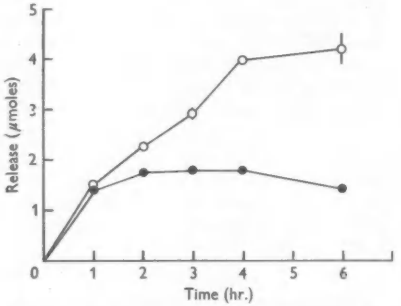

Fig. 4. Release of ninhydrin-reacting material from freshly cut tissue. The tissue (2 g.) was in 10 ml. of water or 0·1 mM-dinitrophenol. The estimated concentration of ninhydrin-reacting material in the tissue was 50 mM. Incubation was in a shaking water bath for 6 hr. at 25°. ●, Water; ○, dinitrophenol. Experiments were performed in duplicate; vertical lines indicate the spread of results (where these exceeded the diameter of the circle).

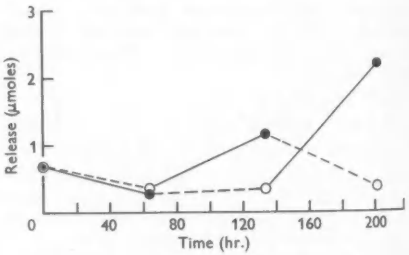

Fig. 5. Effect of alternate aerobic and anaerobic circulation on release of ninhydrin-reacting material. The tissue (2 g.) was in 10 ml. of water or 0·1 mM-dinitrophenol. Incubation under aerobic conditions was for 6 hr. at 25°. Correction was made for small loss into water alone. - - - ○, Period of aerobic circulation; —●, period of anaerobic circulation.

circulated tissue, i.e. tissue with a well-developed active uptake. An extract of 3 hr. was chosen, as this represents the mid-point of the 6 hr. period used to follow uptake. The concentration present after 3 hr. (0·2 mM in ninhydrin-reacting material) would therefore represent the 'average' concentration in the medium resulting from leakage during an uptake experiment with freshly cut tissue.

The results showed that such an extract only slightly inhibited uptake (less than 10 %) by both circulated and freshly cut tissue from the same batch of carrots from which the extract was prepared. This small inhibition of uptake by circulated tissue therefore could not account for the low uptake observed in freshly cut tissue.

These experiments have shown therefore that only a small proportion of the endogenous amino acids leak out of freshly cut tissue, and that the amount released does not account for the low rates of uptake in freshly cut tissue. As can be seen from the previous section the concentration of ninhydrin-reacting material in the water of the tissue tested varied from 30 to 70 mM. It therefore seemed more likely that it was the high internal concentration which was responsible for the inhibition of uptake of amino acids in freshly cut tissue.

Accordingly, 80 % ethanolic extracts and boiling-water extracts of tissue were prepared. These were adjusted to give concentrations of the required order (50 mM with respect to ninhydrin-reacting material) as described in the Materials and Methods section.

Analysis of boiling-water extract and 80 % *ethanolic extract.* Chromatography of these extracts (concentrated to 50 mM in ninhydrin-reacting material) and freshly expressed carrot juice showed the same amino acids to be present in the same relative amounts; in this respect the extracts were therefore substantially representative of the fresh tissue. The major amino acids were glutamine, glutamate, asparagine, aspartate and alanine. These amino acids accounted for most of the ninhydrin-reacting material in the extracts.

The effect of aerobic circulation for 150 hr. on the concentrations of these amino acids in the tissue was studied by visual comparison on paper chromatograms. In one experiment asparagine and the glutamine–aspartate spot increased, whereas glutamate and alanine decreased considerably during the circulation period. In another experiment of 150 hr. all the amino acids decreased to low amounts.

Analysis of the boiling-water extract (50 mM with respect to ninhydrin-reacting material) gave the following results for inorganic ions: Na^+, 47 mM; K^+, 98 mM; PO_4^{3-}, 23 mM.

Effect of extracts on accumulation of D- *and* L-*histidine.* Uptake of histidine has been determined from 0·5 to 6·0 hr. to eliminate the substantial initial inactive uptake from 0 to 0·5 hr. (Birt & Hird, 1956). In several cases, which are noted in the text, the small uptake which is insensitive to dinitrophenol during this period has been subtracted.

Table 2 (Expt. 3) shows that at concentrations of 2·5 and 5·0 mM, the water extract markedly inhibited the accumulation of the two isomers of histidine. It is significant that the percentage inhibition of the D-isomer was very much greater than that of the L-isomer. In comparative experiments shown in Table 2 (Expts. 1 and 2), boiling-water and ethanolic extracts are shown to give quantitatively similar inhibitions of the uptake of L-histidine.

Table 3 shows the active uptake of L-histidine found by using as competitors two amino acids of relatively low and high affinity for the carrier system (glutamine and methionine respectively; Birt & Hird, 1958*b*). Ethanolic and boiling-water

Table 2. *Effect of extracts of carrot tissue on uptake of* D- *and* L-*histidine by aerobically circulated carrot tissue*

Tissue (2 g. fresh weight) was incubated for 6 hr. in 10 ml. of solution at 25°. The initial pH was 5·0 and concentration of D- and L-histidine 5·0 mM. The tissue was circulated aerobically for 46 hr. The uptake period for the extract was 0–6 hr. and, for D- and L-histidine, 0·5–6·0 hr. Different batches of tissue were used in each experiment. At 5 mM, the ninhydrin-reacting material in the ethanolic and boiling-water extracts had a dinitrophenol-sensitive uptake of 19·3 and 14·9 μmoles respectively in the absence of histidine.

Concn. of ninhydrin-reacting material in extract (mM)	Dinitrophenol-sensitive uptake (μmoles)			
	L-Histidine			D-Histidine
	Expt. 1. Ethanolic extract	Expt. 2. Boiling-water extract	Expt. 3. Boiling-water extract	Expt. 3. Boiling-water extract
Nil	19·9	20·2	28·5	17·2
1·0	19·7	17·8	—	—
2·5	15·6	15·9	15·4	2·5
5·0	12·7	12·4	11·4	0·4
10·0	6·9	5·1	—	—

Table 3. *Effect of glutamine, methionine and extracts on uptake of* L-*histidine by aerobically circulated tissue*

Tissue (2 g. fresh weight) was incubated for 6 hr. in 10 ml. of solution at 25°. The initial pH was 5·0 and concentration of L-histidine was 5·0 mM. The tissue was circulated aerobically for 45 hr. and the uptake period of L-histidine was 0·5–6·0 hr.

Competitor	Concn. of ninhydrin-reacting material (mM)	Dinitrophenol-sensitive uptake of L-histidine (μmoles)
None	—	15·1
Ethanolic extract	2·5	11·7
Ethanolic extract	5·0	7·7
Boiling-water extract	2·5	10·2
Boiling-water extract	5·0	7·4
L-Glutamine	5·0	7·4
L-Methionine	2·5	2·1

extracts are included for comparison of effects on the same tissue. The results show that similar inhibitions of L-histidine uptake are produced by 5 mM-glutamine, boiling-water extract, 5 mM with respect to ninhydrin-reactive material, and ethanolic extract, 5 mM with respect to ninhydrin-reactive material. 2 mM-Methionine (with a high affinity) gives greater inhibition than extracts or 5 mM-glutamine.

It has been found that the uptake of ninhydrin-reacting material in these extracts has a large dinitrophenol-sensitive component and that the total uptake is of the order of magnitude obtained with amino acids. It is concluded that the endogenous ninhydrin-reacting material mostly consists of assorted amino acids and this is confirmed by chromatography. As these amino acids would be of the L-configuration, the greater inhibition of uptake of D-histidine as opposed to L-histidine in fresh tissue can be explained in terms of affinity for the carrier.

Birt & Hird (1956) showed that potassium chloride and potassium phosphate buffer decreased the uptake of L-histidine by washed carrot tissue, and the possibility is not excluded that the high concentrations of inorganic ions in the extract inhibit to some extent the uptake of externally supplied amino acids.

DISCUSSION

Birt & Hird (1958*b*), using carrot tissue previously circulated aerobically, showed that uptake of the various amino acids involved a common carrier, and that when these were added together they competed for this carrier. The nature of the side chain was shown to be important in determining the affinity for the carrier, and the L-isomers were shown to have much greater affinity than the corresponding D-isomers. It has also been shown that active uptake increased considerably on circulating the tissue aerobically (Birt & Hird, 1956, 1958*b*).

In the present investigation we have found high concentrations, from 35 to 61 mM, of free amino acids (presumably the L-isomers) in the tissue. During the course of aerobic circulation the uptake of D-amino acids increases from a lower initial value, and to a greater extent than the uptake of the L-amino acids; it has also been shown that the rate of leakage of endogenous amino acids into a medium containing dinitrophenol is lowered on aerobic circulation. Tissue treated in this way when circulated anaerobically shows the converse of these changes. The results obtained are thus consistent with removal of endogenous and competing L-amino acids from the carrier during aerobic circulation and their return during anaerobic circulation. This disappearance and appearance of amino acids at the carrier site is possibly connected with fluxes into and out of the vacuole, but metabolism (Birt & Hird, 1958*a*) of these amino acids will also play a part in their disappearance. Extracts of tissue and comparable amino acid solutions produced inhibitions of the expected magnitude in the uptake of D- and L-histidine by aerobically circulated tissue. It seems likely therefore that the process leading to an increase in the active uptake of amino acids during aerobic circulation is the removal of endogenous and competing material from the carrier site.

The increase in salt-stimulated respiration after aerobic circulation and its loss after anaerobic circulation follows a similar pattern to the accumulation of amino acids under the same conditions. With salt-stimulated respiration, however, the situation is more complex; the reasons for a stimulation of respiration in the presence of potassium chloride are unknown, and the various substrates used will probably make a different quantitative contribution to respiration after aerobic and anaerobic treatments. It is likely therefore that not only might endogenous anions and cations inhibit uptake of the salt in freshly cut and anaerobically circulated tissue, but also that salt-stimulated respiration might only occur with special substrates dominating after aerobic circulation. Consequently it is possible that the development of accumulation of amino acids and of salt-stimulated respiration have in common only the removal of endogenous substances from the region of the carrier (cytoplasm).

There is an extensive literature concerning the accumulation of inorganic ions by plant tissues after aerobic circulation (see review by Sutcliffe,

1959), and a carrier mechanism is generally accepted as being implicated in the accumulation process (see reviews by Epstein, 1956; Sutcliffe, 1959). Rees (1949) has shown that after anaerobic circulation, uptake, by red-beet tissue, of manganese and chloride ions is less than in aerobically treated tissue. Skelding & Rees (1952) and Dale & Sutcliffe (1956) have also shown that extracts of the tissue inhibit the uptake of inorganic ions. There are thus many similarities between this system and the one studied in the present work. These observations on the accumulation of salts after aerobic circulation and the decreased accumulation after anaerobic circulation could similarly have an explanation in terms of the removal or non-removal of competing endogenous anions and cations from the region of the carrier.

The increase in malonate-sensitive respiration by slices of plant tissue after aerobic circulation has obvious parallels with the increase in uptake of amino acids and inorganic ions, and with the increase in salt-stimulated respiration. It has commonly been assumed that the increase in malonate-sensitivity of respiration (Hanly *et al.* 1952; Laties, 1959) is associated with an increase in a qualitatively different component of respiration. However, the problem may have a simpler explanation in terms of the removal, during aerobic circulation, of competitors which prevent the entry of inhibitors, such as malonate, to their sites of action.

SUMMARY

1. The increase in salt-stimulated respiration and of the active accumulation of amino acids in slices of carrot has been investigated during washing by circulation aerobically and anaerobically.

2. Salt-stimulated respiration and amino acid accumulation which increase on aerobic circulation can be reversed by anaerobic circulation of carrot slices.

3. When the rate of dinitrophenol-sensitive accumulation of D- and L-amino acids is compared in freshly cut slices and in aerobically circulated slices, it has been found that the increased rate of accumulation after circulation is much greater with the D-isomer than with the L-isomer.

4. High concentrations (30–70 mM) of free amino acids are found in the tissue fluid, and extracts of the tissue containing these amino acids have been shown to compete with other externally supplied amino acids for the accumulation process.

5. It has been concluded that the increase in salt-stimulated respiration, and in the accumulation of amino acids when the tissue is aerobically circulated, is related to the removal during this process of endogenous substances from the cytoplasm or carrier sites or from both.

6. The results obtained are discussed in relation to the increase in the active accumulation of inorganic ions and of the so called malonate-sensitive respiration.

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Effects of Carbon Dioxide on Mitochondrial Enzymes from *Ricinus*

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Many aspects of the growth and development of plants and of their metabolism are known to be influenced by the carbon dioxide concentration in the surrounding atmosphere (see, for example Crocker, 1948; Harrison, 1953). Notwithstanding the number and variety of these observations, very little is yet known about the effects of carbon dioxide on specific enzyme systems in plants. From his discovery that exposure of plant organs to aerobic atmospheres containing high carbon dioxide concentrations resulted in the accumulation of ethanol and acetaldehyde, Thomas (1925) inferred that some oxidative process was inhibited. However, though carbon dioxide-zymasis (for definition see Thomas, 1925) is induced in many tissues by carbon dioxide in concentrations above about 50 %, it has not been reported in concentrations much below this value (Foster, 1940; Smith, 1957). Walker & Brown (1957) found that the activity of phosphoenolpyruvic carboxylase was decreased by about 30 % in the presence of 10 % of carbon dioxide and that inhibition of the enzyme increased with carbon dioxide concentration until in about 70 % of carbon dioxide the inhibition was virtually complete. The inhibition was shown to be of a non-competitive type and to our knowledge these observations provided the first direct demonstration of the inhibition of an enzyme by carbon dioxide in high concentration.

Succinic acid is known to accumulate in certain plant tissues when they are stored in atmospheres containing 20 % or in some cases 10 % of carbon dioxide (Ranson, 1953; Hulme, 1956; Ulrich & Landry, 1956). The experiments described in this paper were therefore primarily directed towards the investigation of the effects of carbon dioxide concentration on some enzymes involved in the production and consumption of succinate in plant tissues. A convenient source of these enzymes was a particulate preparation from the endosperm of *Ricinus* seedlings, isolated according to the method of Beevers & Walker (1956) and shown by them to exhibit a high tricarboxylic acid-cycle activity.

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The range of carbon dioxide concentrations studied was 0·05–90 %. Whereas concentrations in the upper half of this range may not occur in cells under natural conditions, concentrations in the lower half of the range may not be uncommon. For example, in a well-aerated soil the average carbon dioxide concentration is probably less than 1 %, but in the area around a well-developed root system a concentration of 9 % has been recorded (Russell & Appleyard, 1915), and Leonard (1945) has suggested that the concentration at the surfaces of a root system might on occasions rise to several times this value. In bulky plant organs, e.g. fruits, tubers and stems of trees, where gaseous exchange is impeded, the carbon dioxide concentration is again often very much higher than that of the surrounding atmosphere. The concentrations recorded for the fruits of apple and pear are variable and depend on many factors, including temperature, but average concentrations of 5–10 % within a fruit are common and concentrations nearer 30 % have been recorded (Magness, 1920). Since in such organs the concentration at the centre is presumably higher than that near the surface, carbon dioxide concentrations around 20 % may not be uncommon in the inner zones of many bulky tissues.

It has been suggested that some of the observed effects of high carbon dioxide concentrations in the atmosphere around plant tissues may result from increased acidity within the cells as a result of the solution of carbon dioxide in the sap (see, for example, Small, 1954). Thornton (1933), however, found that saps expressed from a variety of tissues, after storage in high carbon dioxide concentrations, were invariably at a higher pH than saps expressed from similar tissues after storage in air. The increase in pH was ascribed to secondary effects of the carbon dioxide treatment. It is, however, doubtful whether such observations on the overall pH within tissues or cells throw any light on the direct effect of carbon dioxide on the pH within the cytoplasm of cells. The experiments described in this paper were confined therefore to conditions under which the carbon dioxide concentration was varied at a single constant pH value.

A preliminary account of some of this work has already appeared (Ranson, Walker & Clarke, 1957).

MATERIALS AND METHODS

Preparation of mitochondrial fractions. The method was essentially that described by Beevers & Walker (1956). Castor beans (*Ricinus communis* var. Cimarron) were germinated for 4 days in moist, coarse-grade horticultural Vermiculite (heat-expanded mica) at 30° in the dark. The endosperm tissue was then separated and ground in a medium containing 0·5 M-sucrose, 6 mM-$MgCl_2$ and 0·1 M-NaH_2PO_4, and adjusted to pH 7·4 with KOH. The mitochondrial fraction separated by differential centrifuging was washed once and finally resuspended in the grinding medium. The volume of the final suspension was such that 1 ml. contained particles from 10–15 g. of endosperm.

Substrates and cofactors. Stock solutions (0·2 M) of each of the substrate acids were prepared in deionized water and adjusted to pH 7·4 with NaOH. [1-^{14}C]- and [2:3-$^{14}C_2$]-Succinic acid, [1-^{14}C]- and [2:3-$^{14}C_2$]-fumaric acid and sodium [3-^{14}C]pyruvate, purchased from The Radiochemical Centre, Amersham, were diluted with their inactive analogues, dissolved immediately before use and the solutions were adjusted to pH 7·4. Unless otherwise stated the final concentration of each acid in reaction mixtures was 0·01 M.

Diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), coenzyme A (CoA) and cocarboxylase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. These were dissolved in deionized water, adjusted to pH 7·4 and added as required to the reaction mixtures to provide 1 mg. of DPN, 1 mg. of ATP, 0·1 mg. of CoA and 0·5 mg. of cocarboxylase/ml. of mitochondrial preparation used. The final concentrations (mM) of these cofactors in reaction mixtures were approximately 0·4, 0·6, 0·04 and 0·35 respectively.

Regulation of carbon dioxide concentration. Aqueous solutions of $NaHCO_3$ were prepared at molar concentrations calculated on the basis of the Henderson–Hasselbalch equation (see Umbreit, Burris & Stauffer, 1949) to adjust to pH 7·4 at 25° when equilibrated with gas mixtures containing 5, 10, 20 and 30 % of CO_2 in air and 50, 70 and 90 % of CO_2 in O_2. The bicarbonate concentrations were adjusted to allow for dilution on addition to the final reaction mixtures. Gas was bubbled through the solutions for several hours before use.

Activity measurements. Uptakes of oxygen of the particulate preparations in the presence of added substrate acids and cofactors were measured manometrically as described by Beevers & Walker (1956).

Analysis of reaction products. Substrates, cofactors and CO_2–bicarbonate buffer were mixed in either 10 ml. test tubes or 30 ml. flasks fitted with inlets and outlets and the mixtures equilibrated at 25° against gas streams containing the appropriate CO_2 concentration for about 30 min. The particulate preparation was then added and the gas stream continued. In most of the experiments described below, 1 ml. or 2 ml. samples were removed from the final reaction mixture after appropriate time intervals and rapidly added to 20 ml. of boiling 70 % ethanol. The mixture was then concentrated to about 1 ml., adjusted to pH 1 by the addition of HCl, thoroughly stirred into about 15 g. of anhydrous Na_2SO_4 and transferred quantitatively to a Soxhlet thimble. Organic acids were extracted with anhydrous ether in the Soxhlet apparatus for 36–48 hr. The acids were then transferred to a measured amount of water and analysed by chromatography in one or two dimensions on Whatman no. 1 paper with *tert.*-amyl alcohol–formic acid–water (3:1:3, by vol.) and propanol–ammonia (6:4, v/v) as developing solvents (see, for example, Ranson, 1955).

In some of the later experiments with ^{14}C-labelled substrates at a higher specific activity than those used in the earlier experiments, small amounts (5–20 μl.) of the reaction mixture were applied directly to the filter paper and dried rapidly with a hot-air stream to inactivate the enzymes. A mixture of known acids was then added in sufficient amount to allow positive identification by conventional spray techniques on the developed chromatogram of the actual components of the unknown mixtures.

Radioautographs were prepared from the developed chromatograms by placing the paper in contact with Ilfex (Ilford Ltd.) X-ray films for 7 days. Radioactive compounds, located on the paper with the aid of the developed film, were assayed for their ^{14}C content either directly on the paper or after elution by means of a Geiger-counter tube fitted with a thin end-window. Compounds were eluted by placing active areas of the filter paper, cut into pieces about $\frac{1}{2}$ in. square in stoppered tubes with 0·5–2·0 ml. of aq. mN-NH_3 and shaking for 1 hr. on a rotary shaker. Samples (0·3–0·5 ml.) of the ammoniacal extracts were dried on aluminium plates for ^{14}C assay. Self-absorption corrections were applied in all ^{14}C measurements.

RESULTS

The uptakes of oxygen of the various mitochondrial preparations, measured manometrically in the presence of added cofactors and Krebs-cycle acids, were broadly similar to those reported (Beevers & Walker, 1956). Chromatographic analysis of the acid products demonstrated the production of citric acid, *iso*citric acid, α-oxoglutaric acid, succinic acid, fumaric acid and malic acid from their respective precursors in the acid cycle. Succinic acid was consumed with the production of fumaric acid and malic acids by mitochondrial preparations supplemented only with ATP, or without added cofactors.

Effect of carbon dioxide concentration on succinate oxidation

In these experiments the only cofactor added was ATP. Preliminary investigations in which the acids produced from succinate were measured by the spot-area method after paper chromatography suggested that the production of fumarate and malate was markedly inhibited in carbon dioxide–bicarbonate mixtures corresponding to gas concentrations above about 20 %.

To allow more accurate quantitative measurements, [^{14}C]succinate was used in subsequent experiments. In one of these (see legend to Fig. 1), the effects of carbon dioxide in concentrations between 0·05 (air) and 90 % were compared. Samples were removed after time intervals up to 50 min. and inactivated with boiling 70 % ethanol. In the ether-soluble constituents of these samples

the recovery of the ^{14}C supplied originally as succinate was uniformly better than 90%. The sum of the ^{14}C contents of well-defined radioactive components when counted directly on chromatograms varied on individual sheets between 75 and 105%. Since there was no apparent correlation between the percentage recovery and previous carbon dioxide treatment, the low recoveries are attributed to imperfections in technique rather than to loss of labile components during chromatography.

Visual examination of the radioautographs showed that in air containing up to 10% of carbon dioxide there was a steady depletion of succinate with a corresponding increase in malate and fumarate, whereas in 20% and higher concentrations of carbon dioxide consumption of succinate was slight. In addition to the labelled acids mentioned, other labelled components were present on chromatograms but none of these corresponded with the known positions of Krebs-cycle intermediates. Three of the unidentified compounds contained, in certain instances, considerable proportions of the total ^{14}C activity and these are referred to below as unknowns A, B and C. Their approximate co-ordinates relative to malic acid (100) were, for the alkaline solvent, A, 217; B, 225; C, 340; and, for the acid solvent, A, 70; B, 88; C, 61. As an example, counts made from a two-dimensional chromatogram of the products after 50 min. in air gave the following figures: succinate 900, malate 1600, fumarate 250, A 180, B 200 and C 100 counts/min., and there were other less-active components. After a 50 min. period in 70% of carbon dioxide most of the ^{14}C remained in succinate, but unknown compounds A, B and C were still detectably labelled.

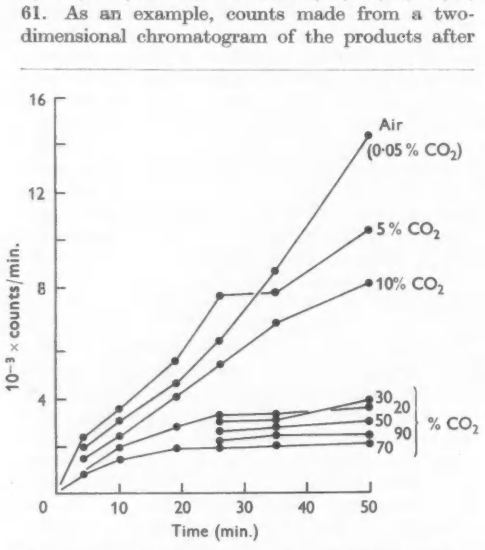

Fig. 1. [^{14}C]Malate produced from [^{14}C]succinate by mitochondria in equilibrium with atmospheres containing from 0·05 to 90% of CO_2. Reaction mixtures contained 3 ml. of mitochondrial suspension, 6·9 mg. of ATP, 8 mg. of succinate containing 10 μc of [1-^{14}C]succinate, and CO_2–bicarbonate mixture and water as required. The final volume was 6·9 ml., temperature 25° and pH 7·4.

Because of the production of these unidentified compounds from succinate, the activity appearing in malic acid appeared to be the safest measure of the oxidative consumption of succinate by way of succinic oxidase and fumarase, and the ^{14}C present in malic acid after various time intervals in gas atmospheres containing different carbon dioxide concentrations is recorded in Fig. 1. In 10% of carbon dioxide there was, overall, a 40% decrease in the rate of malate production. In higher concentrations malate production was more markedly retarded from the start and virtually stopped completely within about 25 min. The rate of malate production was faster initially in 5% of carbon dioxide than in air. A similar result was observed in one other experiment but its significance remains in doubt. [^{14}C]Fumarate was detected whenever labelled malate was produced but at amounts never greater than 25% of the malate. No label was detected in citrate in any gas concentration, though in parallel experiments in which the reaction mixtures contained DPN, CoA and cocarboxylase in addition to ATP, labelled citrate was readily detected after about 26 min. in air.

Since the major part of ^{14}C supplied to the mitochondrial preparation was accounted for on many of the chromatograms it appeared probable that malate produced was not converted into oxaloacetate or pyruvate to any marked extent, and that the malate accumulating represented substantially that produced from succinate. The conclusion drawn was that the oxidative consumption of succinate to yield malate was markedly inhibited in carbon dioxide concentrations of 20% and above. In the higher concentrations where malic acid production was retarded there was no accumulation of fumarate. It thus appeared probable that it was a component of the succinic oxidase which was sensitive to carbon dioxide.

Carbon dioxide concentration and fumarate consumption

When mitochondrial preparations were supplied with [1:4-$^{14}C_2$]fumarate in the presence of ATP only, in experiments otherwise similar to those described in the previous section, the fumarate was converted into malate at rates which were the same in high carbon dioxide concentrations as in air. Small amounts of ^{14}C were detectable again in several unidentified components but no other stable Krebs-cycle intermediate was labelled.

Similar experiments in which the mitochondria were supplied with the four cofactors were carried out with [1:4-$^{14}C_2$]- and [2:3-$^{14}C_2$]-fumarate as substrates. In air the carboxyl-labelled acid yielded labelled citrate and *iso*citrate but no labelled succinate, whereas the fumaric acid labelled in the central carbon atoms yielded labelled citrate, *iso*citrate and, eventually, labelled succinate. These results are in accord with those which would be expected from present knowledge of the partial reactions of the Krebs cycle. Again the carbon dioxide concentration had no effect on the conversion of fumarate into malate.

Carbon dioxide concentration and pyruvate oxidation

Mitochondria supplemented with the four cofactors were supplied with [3-^{14}C]pyruvate in reaction mixtures in equilibrium with air, and 30 and 70 % of carbon dioxide (see legend to Table 1). Unlabelled citric acid, malic acid, succinic acid and fumaric acid were included in the mixtures to serve as traps for their labelled analogues produced from the pyruvate. Samples were removed at intervals up to 90 min. and applied directly to origins on paper chromatograms. In developing the chromatograms the sheets were allowed to remain in the alkaline solvent for a sufficiently long time for residual pyruvate to run off the paper. They were then developed in the second direction in the acid solvent. The only labelled components present in other than trace amounts which could be detected on any sheets were citric acid, *iso*citric acid, malic acid, succinic acid, fumaric acid and alanine. The appearance of labelled alanine was attributed to the presence of an amino acid and of a transaminase capable of transferring an amino group from the acid to the labelled pyruvate. The presence of transaminases in the mitochondrial preparation had been demonstrated previously (D. A. Walker, unpublished work).

In air after 5 min. the ^{14}C was present mainly in citric acid. With increase in time malic acid was labelled, and after 90 min. contained more ^{14}C than the citric (+*iso*citric) acid. Succinate accumulated ^{14}C slowly (see Table 1). In 30 % of carbon dioxide the incorporation of ^{14}C from the pyruvate was markedly retarded and after 90 min. no measurable activity was present in either fumarate or malate. In 70 % of carbon dioxide the incorporation of ^{14}C was very slight and the only compound (other than alanine) containing appreciable activity after 90 min. was citrate. Taking as a rough measure the total amount of ^{14}C present after 90 min. in the Krebs-cycle acids detected on chromatograms, the relative amounts of pyruvate metabolized in air, 30 and 70 % of carbon dioxide were about 100, 24, and 5 respectively. The total activity present in the acids in air after 90 min. represented about 40 % of the ^{14}C supplied originally. This fraction represents a minimum estimate of the pyruvate consumed.

The results described earlier indicate that the succinic oxidase would be markedly inhibited in both 30 and 70 % of carbon dioxide, and this inhibition might be expected to retard in time the utilization of pyruvate in Krebs-cycle reactions. The presence of activity in succinate after 90 min. in 30 % of carbon dioxide with no detectable trace in fumarate and malate points to the inhibition of the succinic oxidase in this experiment also. If, however, this oxidase were the only partial system inhibited, one might expect an accumulation of ^{14}C in the succinate to an extent greater than that which occurred in air and, moreover, the accumulation of ^{14}C in citric acid and other acids, e.g. α-oxoglutaric acid, which precede succinic acid in the cycle. Such accumulations were not observed. Moreover, the inhibition of pyruvate metabolism was greater in 70 % than in 30 % of carbon dioxide, which appears unlikely if succinic oxidase were the sole enzyme system affected. These results suggest therefore that in 30 and 70 % of carbon dioxide some enzyme involved in the production of citrate from pyruvate was markedly affected in addition to the succinic oxidase.

Table 1. *Incorporation of* [^{14}C]*pyruvate into acids by mitochondrial preparations*

Values of ^{14}C are in 10^{-3} × counts/min. Reaction mixtures contained in 1 ml. of mitochondrial suspension: 1 mg. each of DPN, ATP, cocarboxylase, 0·1 mg. of CoA, 1 mg. each of succinate, fumarate, L-malate, citrate, 1·6 mg. of pyruvate containing 30 μc of [3-^{14}C]pyruvate and CO_2–bicarbonate mixture and water as required. The final volume was 3 ml., temperature 25° and pH 7·4. No entry (–) in the table indicates activities less than $1·5 \times 10^3$ counts/min.

	Air		30 % CO_2		70 % CO_2	
	30 min.	90 min.	30 min.	90 min.	30 min.	90 min.
Citric acid + *iso*citric acid	105	198	21	93	—	20
Succinic acid	—	18	—	15	—	—
Malic acid	15	225	—	—	—	—
Fumaric acid	2	24	—	—	—	—

DISCUSSION

In the previous section results have been described which demonstrate the sequential production of citric acid, *iso*citric acid, α-oxoglutaric acid, succinic acid, fumaric acid, and malic acid in mitochondrial preparations supplied with ATP, DPN, coenzyme A, cocarboxylase and suitable substrates. Moreover, in similar preparations with added cofactors and acid substrates labelled with ^{14}C the oxidative production of succinate from fumarate and of malate from pyruvate has been demonstrated. These results, together with previous manometric observations (Beevers & Walker, 1956, Walker & Beevers, 1956), are in keeping with the now well-established view that intact mitochondria can bring about the integrated reactions of the Krebs cycle. Particulate preparations from *Ricinus* are well suited to the study of these reactions in cell-free systems particularly since their activity is extremely high and their endogenous respiration negligible. The production in the particulate preparations of alanine from pyruvate would appear to confirm the presence of some transaminase activity. The production of the unknown compounds A, B and C (if in fact these compounds are not artifacts of the analytical technique) may indicate the presence of other enzymic activities. Most of the results described in this paper are, however, concerned with a comparison between the rates of several of the partial reactions in mixtures in equilibrium with air and with aerobic atmospheres containing various concentrations of carbon dioxide. For simplicity in this section we shall continue to refer results to the carbon dioxide concentration though it is possible that the operative factor is the bicarbonate ion in equilibrium with the carbon dioxide.

The main observation arising was that the conversion of succinate into malate was markedly affected by carbon dioxide in concentrations above about 10 %. Since the conversion of fumarate into malate was apparently unaffected by such concentrations the conclusion drawn is that the succinic oxidase was the enzymic system inhibited. The results summarized in Fig. 1 indicate therefore that the oxidase was inhibited to the extent of about 40 % by an atmosphere containing 10 % of carbon dioxide and virtually completely inhibited by atmospheres containing 20 % or more of carbon dioxide, under the particular conditions of this experiment. Work by Bendall, Ranson & Walker (1958, 1960) suggests, however, that the degree of inhibition may vary with the concentration of succinate in the system.

The experiments on the oxidative consumption of pyruvate were of a more exploratory nature. Nevertheless, they suggest that in 30 % of carbon dioxide, though succinate consumption was apparently completely inhibited, the incorporation of pyruvate into citrate continued for some time but at a much slower rate than in air. In 70 % of carbon dioxide, the incorporation of pyruvate into citrate was very slow. One interpretation is that pyruvic oxidase or possibly condensing enzyme is sensitive to carbon dioxide but that complete inhibition occurred at a higher concentration than that required to inhibit succinic oxidase to the same degree.

Although at this stage attempts to relate the effects of atmospheres containing enhanced carbon dioxide concentrations on intact plant tissues to those observed with the particulate preparation must be cautious, it appears probable that the accumulation of succinate in plant organs stored in the presence of 10–20 % of carbon dioxide can be attributed to an inhibition of succinic oxidase in those organs. Further, for plant tissues in which respiratory oxidations are mediated by mitochondrial enzymes the inhibition of succinic oxidase and possible other cycle enzymes by carbon dioxide may in part explain some of the reported effects of high concentrations of this gas on a variety of metabolic processes in plants.

An attempt to determine the site of the carbon dioxide inhibition in the succinic-oxidase system is reported elsewhere (Bendall *et al.* 1958, 1960).

SUMMARY

1. Reactions of the Krebs cycle in a mitochondrial preparation from *Ricinus* endosperm have been studied in air and in carbon dioxide concentrations up to 90 %.

2. Succinic oxidase is markedly inhibited in carbon dioxide concentrations of 10 % and above. At higher concentrations the conversion of pyruvate into cycle acids is also depressed, suggesting an inhibitory effect of carbon dioxide on pyruvic oxidase or condensing enzyme.

3. The results are discussed in relation to the effects of carbon dioxide on intact plant tissues under aerobic conditions.

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Effects of Carbon Dioxide on the Oxidation of Succinate and Reduced Diphosphopyridine Nucleotide by *Ricinus* Mitochondria

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The oxidation of succinate to fumarate by particulate preparations from *Ricinus* endosperm is retarded in atmospheres containing more than about 10% of carbon dioxide (Ranson, Walker & Clarke, 1957, 1960) and the retardation has been attributed to an inhibition of the succinic-oxidase system of the mitochondria. The present paper is an enlargement of a preliminary account (Bendall, Ranson & Walker, 1958) and describes the results of an investigation to determine which components of the respiratory chain are inhibited. The effects of carbon dioxide–bicarbonate mixtures on the succinic–cytochrome *c* reductase system and on cytochrome *c* oxidase were determined, and evidence is presented that the actual site of inhibition is succinic dehydrogenase.

Since previous work had also suggested that the enzymic reoxidation of reduced diphosphopyridine nucleotide may be affected by high concentrations of carbon dioxide, the effects of carbon dioxide–bicarbonate mixtures on the reduced diphosphopyridine nucleotide–cytochrome *c* reductase system were also determined.

In an attempt to assess the effects of the sodium bicarbonate in the mixtures with carbon dioxide the effects of some other salts have also been studied.

MATERIALS AND METHODS

Cytochrome *c* was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. and Boehringer und Söehne, Mannheim, Germany. It was dissolved in 1% (w/v) 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4, and estimated spectrophotometrically at 550 mμ. Molar extinction coefficients of $27{\cdot}7 \times 10^3$ l. moles^{-1} cm.$^{-1}$ for the reduced form and $9{\cdot}2 \times 10^3$ l. moles^{-1} cm.$^{-1}$ for the oxidized form were used (Margoliash, 1954). Reduced cytochrome *c* was prepared by adding a slight excess of sodium dithionite and bubbling O_2 through the solution for 5 min. to remove the excess of dithionite. Reduced diphosphopyridine nucleotide (DPNH) was purchased from the Sigma Chemical Co.

Preparations of mitochondrial fractions. Suspensions of mitochondria from the endosperm of castor beans (*Ricinus communis* var. Cimarron) were prepared as described previously (Ranson *et al.* 1960). 30 min. before spectrophotometer experiments the suspensions were diluted tenfold with ice-cold demineralized water, since it has been found that *Ricinus* mitochondria will not react easily with added cytochrome *c* when suspended in 0·5 M-sucrose but react readily after incubation in 0·05 M-sucrose. This is attributed to the osmotic rupture of membranes which would otherwise limit the free access of cytochrome *c* to enzymic centres.

Enzyme assays. Succinic–cytochrome *c* reductase, DPNH–cytochrome *c* reductase and cytochrome *c* oxidase activities were measured spectrophotometrically by

methods already described (Bendall *et al.* 1958) with a Unicam SP. 500 spectrophotometer in a temperature-controlled room at 25°.

Spectroscopic observations. These were made with a Zeiss microspectroscope ocular. The scale was set with the α-band of reduced cytochrome *c* at 550 mμ.

Regulation of carbon dioxide concentration. Conditions in the aqueous reaction mixtures corresponding to various atmospheric CO_2 concentrations were simulated by the addition of CO_2–bicarbonate mixtures as described (Ranson *et al.* 1960). The procedure was modified slightly for the spectrophotometer experiments in that the CO_2 concentration was regulated simply by the addition of the appropriate quantity of 0·316 M-$NaHCO_3$ which had previously been brought to equilibrium at pH 7·4 and 25° with a gas mixture containing 90% of CO_2 and 10% of O_2. The bicarbonate was added to the other components in closed spectrophotometer cells immediately before the reactions were started. Subsequent determinations of initial rates were completed within 2 min. Previous experience (Walker & Brown, 1956) had indicated that the loss of CO_2 from solution in these circumstances is extremely small.

RESULTS

Preliminary observations

The effects of a carbon dioxide–bicarbonate mixture on the succinic-oxidase system were examined spectroscopically as follows. To a 1 cm. cell containing 1·6 ml. of a suspension of washed mitochondria was added 0·4 ml. of 0·34 M-sodium bicarbonate saturated with carbon dioxide to give a final concentration corresponding to approximately 20% of carbon dioxide. A control cell was set up in which 0·34 M-sodium chloride replaced the bicarbonate solution. The cells were shaken with air and to each was added 0·5 ml. of 1·2 M-potassium succinate. In the control mixture the α-bands of reduced cytochromes *a*, *b* and *c* rapidly became visible at 603 mμ, 562–563 mμ and 551–552 mμ respectively. The α-bands of cytochromes *a* and *c* appeared almost simultaneously closely followed by the α-band of cytochrome *b*. The complete reduction took about 15 sec. In the cell containing bicarbonate the order of reduction of the cytochromes was the same, but the rate was slower with complete reduction being attained in about 30 sec. In both cases the cytochromes were reoxidized on shaking in air. The addition of a further 0·4 ml. of carbon dioxide–bicarbonate mixture increased the time for complete reduction to 45 sec., whereas the addition of a corresponding amount of sodium chloride to the control was without effect. Again in both cases the cytochromes were reoxidized on shaking with air.

The suggestion from these observations that the inhibition by bicarbonate or carbon dioxide occurred at a point below cytochrome *b* in the respiratory chain was consistent with the following qualitative observations. In an evacuated Thunberg tube the reduction of methylene blue by the mitochondria, with succinate as substrate, was markedly inhibited in the presence of carbon dioxide–bicarbonate mixture at a final concentration corresponding to 30% of carbon dioxide. No change was detectable with the microspectroscope in the spectrum of reduced cytochrome *c* in the presence of a high concentration of the carbon dioxide–bicarbonate mixture. There was no detectable change in the appearance of the α-band of cytochrome *a* when, in an evacuated Thunberg tube, sodium bicarbonate was tipped from the side arm into a reaction mixture containing mitochondria and succinate, to yield a final concentration of 0·6 M.

Spectrophotometric measurements

The effects of various concentrations of bicarbonate–carbon dioxide buffer on the succinic- and DPNH–cytochrome *c* reductase systems and on cytochrome *c* oxidase are shown in Figs. 1–3. The activity of succinic–cytochrome *c* reductase fell off rapidly with increasing carbon dioxide concentration above 5%, as little as 12% of carbon dioxide causing a 50% inhibition. DPNH–cytochrome *c* reductase was also inhibited by carbon dioxide concentrations above about 30% but the activity fell off less rapidly than that of succinic–cytochrome *c* reductase and appreciable inhibition occurred only at relatively high carbon dioxide concentrations. The carbon dioxide concentration required for 50% inhibition was about 60%. On the other hand, cytochrome *c* oxidase showed a marked increase in activity at carbon dioxide concentrations

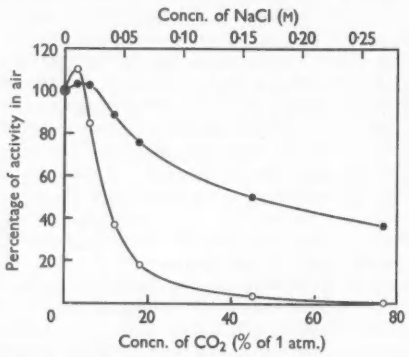

Fig. 1. Effect of CO_2–bicarbonate mixtures (○) and of NaCl (●) on the succinic–cytochrome *c* reductase system. The reaction mixtures contained, in a final volume of 3 ml.: 0·67% of tris–HCl buffer, pH 7·4, 31 μM-cytochrome *c*, 0·5 mM-KCN, pH 7·4, 20 mM-sodium succinate, enzyme and the appropriate quantity either of 0·316 M-$NaHCO_3$, which had been equilibrated against $CO_2 + O_2$ (90:10) at 25°, or of 0·316 M-NaCl. Temp. 25°.

around 20% but at higher concentrations the activity declined and above 60% of carbon dioxide the activity was markedly below that in air. The degree of stimulation observed in different preparations was rather variable, and in one case the maximum activity was double that in the absence of carbon dioxide.

In order to obtain further information about the types of inhibition involved with the cytochrome reductases the rates of reaction were measured at different concentrations of succinate or DPNH for a series of carbon dioxide concentrations. In Figs. 4 and 5 the results have been plotted according to the method of Lineweaver & Burk (1934) for the succinic– and DPNH–cytochrome *c* reductases respectively. It seems clear that the inhibition of succinic–cytochrome *c* reductase was essentially of the competitive type, whereas that of DPNH–cytochrome *c* reductase was non-competitive. However, the Lineweaver–Burk plots for the succinic system passed through a point which lay a little off the $1/v$ axis, which suggests that there may have been a small contribution from a non-

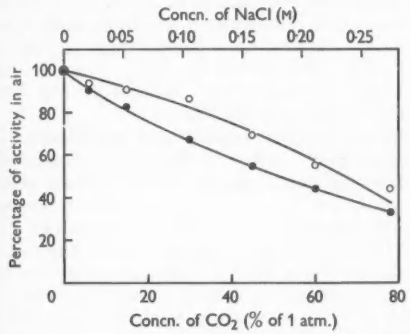

Fig. 2. Effect of CO_2–bicarbonate mixtures (○) and of NaCl (●) on the DPNH–cytochrome *c* reductase system. The reaction mixtures were as given in Fig. 1 except that sodium succinate was replaced by 0·129 mM-DPNH. Temp. 25°.

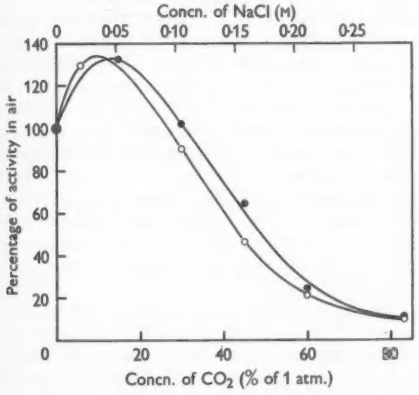

Fig. 3. Effect of CO_2–bicarbonate mixtures (○) and of NaCl (●) on cytochrome oxidase. The reaction mixtures contained, in a final volume of 3 ml.: 0·67% of tris–HCl buffer, pH 7·4, 14·9 μM-reduced cytochrome *c* and the appropriate quantity of either CO_2–bicarbonate mixture or NaCl as in Fig. 1. Temp. 25°.

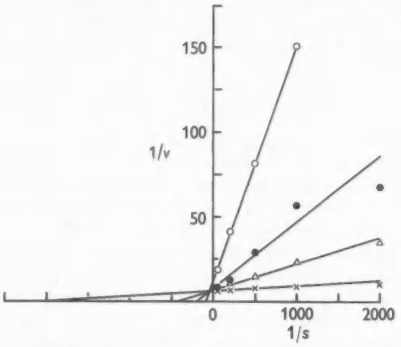

Fig. 4. Effect of succinate concentration on succinic–cytochrome *c* reductase at different concentrations of CO_2–bicarbonate buffer. *v*, reaction velocity (μmoles of cytochrome *c*/min./g. of fresh tissue); *s*, succinate concn. (M). ○, 17·3% of CO_2; ●, 8·7% of CO_2; △, 4·3% of CO_2; ×, no CO_2. Other reaction conditions were as given in Fig. 1.

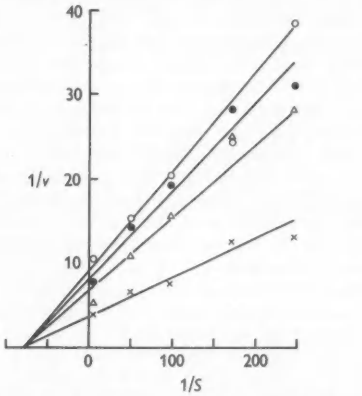

Fig. 5. Effect of DPNH concentration on DPNH–cytochrome *c* reductase at different concentrations of CO_2–bicarbonate buffer. *v*, reaction velocity (μmoles of cytochrome *c*/min./g. of fresh tissue); *S*, DPNH concn. (mM). ○, 78% of CO_2; ●, 60·6% of CO_2; △, 43·4% of CO_2; ×, no CO_2. Other reaction conditions were as given in Fig. 2.

competitive type of inhibition (Dixon & Webb, 1958). This could have been due to a non-specific salt effect of the sodium bicarbonate since sodium chloride also gave some inhibition at low concentrations (Fig. 1).

The competitive nature of the inhibition of the succinic system indicates that the inhibition is reversible. The following observations suggest that the inhibitions of the other enzymic systems by high concentrations of carbon dioxide and bicarbonate are also reversible. Two samples of the original mitochondrial preparation were diluted tenfold, one by the addition of water, the other by the addition of carbon dioxide–bicarbonate buffer to give a final mixture in equilibrium with 80 % of carbon dioxide. They were allowed to stand in the cold for 30 min. Cytochrome oxidase and DPNH–cytochrome *c* reductase activities were then assayed in reaction mixtures containing 0·1 ml. of the treated mitochondrial preparations in a final volume of 3 ml. If the combination of carbon dioxide or bicarbonate with the enzymes had been irreversible the activities in the preparations treated with carbon dioxide–bicarbonate would have been very markedly inhibited. In fact the activities of DPNH–cytochrome *c* reductase and of cytochrome *c* oxidase in the preparation treated with carbon dioxide–bicarbonate mixture were 99 and 114 % respectively of those in the sample diluted with water.

Effects of salts other than bicarbonate

To test the specificity of the inhibitions by carbon dioxide–bicarbonate mixtures, the effects of salts other than sodium bicarbonate on the three enzymic systems were investigated. The effects of sodium chloride on the DPNH–cytochrome *c* reductase and on the cytochrome oxidase were clearly similar to those of sodium bicarbonate (Figs. 2, 3). Inhibitory effects were obtained also with other salts, e.g. the percentage inhibitions of the reductase by sodium bicarbonate, sodium chloride, potassium chloride and sodium sulphate (each 0·274 M) and by 0·137 M-sodium sulphate were 56, 67, 63, 78 and 59 % respectively. In contrast the inhibition of the succinic–cytochrome *c* reductase by sodium chloride was markedly less than by bicarbonate (Fig. 1).

The comparisons of the effects of sodium chloride and sodium bicarbonate–carbon dioxide mixture shown in Figs. 1, 2 and 3 are for equivalent concentrations of sodium ion.

DISCUSSION

The inhibitions of the succinic and DPNH oxidase systems by carbon dioxide–bicarbonate mixtures appear to be of two types. The first kind of inhibition affected the succinic–cytochrome *c* reductase system only, and it was appreciable at concentrations of carbon dioxide below 20 %. The fact that the inhibition was competitive with respect to succinate and that sodium chloride had only a small effect on the system in the equivalent concentration range both indicate that this type of inhibition is a specific effect of the carbon dioxide mixture. The competitive nature of the inhibition also suggests that the actual component of the system affected was succinic dehydrogenase. This interpretation is consistent with the observations in the spectroscope and Thunberg experiments which indicated that the inhibition occurs at some point below cytochrome *b* in the succinic-oxidase chain. They provided no evidence of an inhibition at any point in the chain between cytochrome *b* and cytochrome *a*. Whether the inhibition was due to carbon dioxide or bicarbonate or possibly to both components of the mixture is at present unknown.

The second kind of inhibition is a relatively non-specific effect of high salt concentration which affected all three enzyme systems studied. The similarity of the effects of sodium chloride and carbon dioxide–bicarbonate mixtures on the DPNH–cytochrome *c* reductase and cytochrome *c* oxidase systems suggests that the effects of the solutions containing high carbon dioxide concentrations on those systems was probably due not to carbon dioxide itself but to the high sodium bicarbonate concentration. It is not yet clear whether this was an effect due to the anion or the cation. The specific inhibition of the cytochrome oxidase of root mitochondria by bicarbonate observed by Miller & Evans (1956) was not observed under the different experimental conditions of the present work.

From the foregoing it appears that the marked inhibition of succinic oxidase in the intact mitochondria (Ranson *et al.* 1960) in bicarbonate mixtures in equilibrium with carbon dioxide concentrations above about 10 % can be attributed very largely to inhibition of the succinic-dehydrogenase component. The further inference from the earlier experiments that pyruvic oxidase may be inhibited at still higher concentrations of carbon dioxide and bicarbonate may possibly relate to the inhibition of DPNH–cytochrome *c* reductase and cytochrome *c* oxidase by the bicarbonate.

To what extent all of these results are relevant to the effects observed when intact plant tissues are exposed to atmospheres containing high concentrations of carbon dioxide is at present uncertain. It seems probable, however, that the accumulation of succinate noted in a variety of plant tissues after storage in atmospheres containing 10–20 % of carbon dioxide (Ranson, 1953; Hulme, 1956; Ulrich & Landry, 1956) is related to an inhibition of succinic dehydrogenase in the tissues. The observation that the inhibition of this enzyme by carbon

dioxide is of a competitive nature would account for such variation as occurs in the level of carbon dioxide required to bring about succinate accumulation in different plant tissues.

Whether or not very high concentrations of carbon dioxide in the atmosphere around a plant tissue could in fact result in a sufficiently high bicarbonate concentration in the cytoplasm to bring about the inhibition of DPNH–cytochrome *c* reductase and cytochrome *c* oxidase is not known. If this did occur the combined inhibition of these enzymes together with that of the succinic dehydrogenase would go a long way towards explaining the onset of carbon dioxide zymasis (Thomas, 1925) in atmospheres containing carbon dioxide in concentrations approaching 50% and abundant oxygen.

SUMMARY

1. The rates of three reactions concerned in the oxidation of succinate and reduced diphosphopyridine nucleotide by *Ricinus* mitochondria were determined in reaction mixtures in equilibrium with various concentrations of carbon dioxide at pH 7·4 and 25°.

2. Succinic–cytochrome *c* reductase was markedly inhibited by relatively low concentrations of carbon dioxide–bicarbonate but only slightly by corresponding concentrations of sodium chloride. The inhibition was competitive with respect to succinate concentration. For this and other reasons it is inferred that the carbon dioxide-sensitive component of the reductase is succinic dehydrogenase.

3. Reduced diphosphopyridine nucleotide–cytochrome *c* reductase was inhibited by high concentrations of sodium bicarbonate and other salts.

4. Cytochrome *c* oxidase was stimulated by low concentrations and inhibited by high concentrations of sodium bicarbonate and other salts.

5. The results have been discussed in relation to the observed effects of carbon dioxide on the oxidation of succinate and other acids by intact mitochondria and the effects of carbon dioxide in the environment around intact plant tissues.

The work described in this paper was started and carried out for the most part in the Department of Botany, King's College, Newcastle. The authors wish to express their thanks to Professor M. Thomas, F.R.S., for his continued interest and assistance. Several experiments were also carried out with apparatus financed by the D.S.I.R. and University of London, Central Research Fund. We are also grateful for a quantity of *Ricinus* seeds provided by the Baker Castor Oil Co., San Diego, Calif., U.S.A. One of us (D.S.B.) is indebted to the Agricultural Research Council for a research studentship held while part of the work was in progress.

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The Measurement of the Diffusion of Oxygen through Respiring Tissue

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The diffusion coefficient of oxygen through non-respiring tissue has been measured by Krogh (1919) The figure he obtained was a little lower than that through water. Similar values have been obtained by other workers (Kirk & Johnsen, 1951). In general the diffusion coefficient of oxygen through tissue appeared to be equal to that through water multiplied by the fraction of the tissue composed of water. It seemed that the transport of oxygen through tissues consisted of simple diffusion through the aqueous part uninfluenced by the solid part. That this is not universally true was shown by Longmuir & Roughton (1952), who found that the diffusion coefficient of nitrogen and carbon monoxide through haemoglobin solutions of the concentration found in red cells was about one-

tenth of that through water. We have found some evidence to suggest that there might be an increase in the diffusion coefficient of oxygen through respiring tissue, perhaps as a result of 'protoplasmic streaming'. It was necessary therefore to determine the diffusion coefficient of oxygen through respiring tissue. This has been done by Hill (1928), who measured the uptake of oxygen by anoxic muscle and obtained a figure similar to Krogh's. There were two possible sources of error. If the affinity of the tissue for oxygen was not great enough, Hill's equation might not be valid, and he was measuring the diffusion of oxygen through tissue that had been anoxic. Seifriz & Epstein (1941) have shown that 'protoplasmic streaming' is not resumed until some time has elapsed after the application of an inhibitory influence.

The application of Warburg's equation (Warburg, 1923) to the measurement of the diffusion coefficient of oxygen through respiring tissue has been considered previously (Longmuir, 1958; Longmuir & Bourke, 1959).

Warburg's equation states that

$$C_1 = C_0 - \frac{A}{D}\frac{d^2}{8},$$

where C_1 is pO_2 in the deepest cell layer of the slice; C_0, pO_2 at the surface of the slice; A, oxygen uptake of the slice in ml./min./ml. of tissue; D, diffusion coefficient expressed as ml. of oxygen/cm.^{2}/min. with a partial pressure gradient of 1 atm./cm.; d, slice thickness. This equation assumes that the respiration rate is independent of the partial pressure of oxygen. It has been shown (Longmuir, 1957) that this is true for cells and mitochondria above the critical oxygen concentration (the critical oxygen concentration is the lowest concentration that will produce the maximum respiration rate). In the present work we have found that it is true also for tissue slices. It seems reasonable to assume that when any part of the slice contains less oxygen than is necessary to maintain the maximum respiration rate of that region the slice as a whole will respire at less than its maximum rate. In other words, the critical oxygen concentration for the slice (C_s) will occur when the critical oxygen concentration for the terminal oxidase (C_c) in the middle of the slice has been reached. Thus it is possible to take C_s and C_c as particular values of C_0 and C_1 respectively. Below C_s Warburg's assumption does not hold and presumably his equation is invalid. We wished to ascertain if it is valid above this value. We found that the C_s of liver slices was about 20 times as great as the C_c of individual cells, so as a first step we neglected C_c and Warburg's equation reduced to

$$C_s = \frac{A}{D}\frac{d^2}{8}.$$

If this is true then C_s should increase as the square of the slice thickness. Experimentally we found if we increased the thickness from 0·1 to 0·5 mm., C_s increased at most only twofold, instead of the 25-fold rise required by this equation.

This finding might have been due to a structural factor; for example, the correct value of d to use in the equation might have been the thickness of the liver muralium. However, we obtained the same kind of results with liver, kidney and heart slices. As the histological pattern of all these tissues is different it seems unlikely that we are concerned with a problem of tissue architecture.

It is known that the metabolism of individual liver cells is different from that of slices (Laws & Stickland, 1956), so we were probably not justified in assuming that the C_c for cells in the slice is identical with that of individual cells and that therefore this figure should not be neglected. Warburg's equation could be rewritten as

$$d^2 = \frac{8DC_s}{A} - \frac{8DC_c}{A}.$$

If this equation is valid then a plot of d^2 against C_s/A should yield a straight line of slope $8D$ and intercept on the y axis of $-8DC_c/A$. The intercept on the x axis should be C_c/A. The experiments to be described were designed to determine to what extent the relationship between d^2 and C_s/A could be regarded as linear. If linearity holds then the value of the diffusion coefficient of oxygen through respiring tissue could be determined, and also the C_c of that terminal oxidase which has the lowest affinity for oxygen

EXPERIMENTAL

Solutions. The slices were suspended in a solution of the following molarities: 0·05 M-KCl; 0·05 M-potassium phosphate buffer, pH 7·3, 0·12 M-potassium succinate. The water used was glass distilled, since it had been found that deionized water contained the substances responsible for the electrode poisoning found in earlier work (Longmuir, 1954). The slices respired fairly well in the absence of succinate, but the respiration rate decreased a little with time. The object of this work was to determine the oxygen concentration at which the respiration rate began to diminish, so it was necessary to ensure that the Q_{O_2}, in the presence of enough oxygen, did not fall. This was achieved by adding succinate. It was found that a high concentration (0·12 M) was the minimum necessary to produce the maximum Q_{O_2} in the thickest slices used. The solution has the same freezing point as serum so it is presumably isosmotic.

Slices. These were cut with the McIlwain tissue slicer (McIlwain & Buddle, 1953). The tissues were obtained from 6-week-old rats. Slices of liver were cut from near the periphery of a lobe in order to avoid regions containing large blood vessels. At the same time very narrow slices were discarded as in the derivation of Warburg's equation diffusion

from the edges is neglected. Before cutting the kidney slices the organ was decapsulated and bisected. Heart-muscle slices were cut from the wall of the left ventricle.

The thickness of an individual slice was determined by dividing its weight by the specific gravity and the area. The area was measured by cutting out a piece of Paxalin of the same area as the slice. From the weight of this and its area/unit wt., the area of the slice was calculated. We found that the slices were slightly thinner than expected from the setting of the ratchet on the tissue slicer. The thickness of the slices did not alter during the period of measurement in the polarographic cell.

Methods

The polarographic method used for measuring oxygen concentrations and respiration rates is essentially that of Longmuir (1957) but with some modifications. It was necessary to file the gold electrode flush with the glass to prevent the slices becoming entangled. It was found possible to introduce the slices through the side arm but, as this obviously distorted them, we placed them in the solution in the cell before introducing the rubber disk and electrode. Measurements of oxygen concentration were carried out at 37°. We made the following observations: (1) Except in a few experiments the slices remained intact despite the rapid rotation of the electrode. When the slices did break up they did so at once and those experiments were abandoned. If the slices survived the first few minutes they remained intact for the rest of the period of measurement. (2) Provided that not too many slices were put in the cell at once they did not tangle with one another; nor did they produce any 'noise' on the recorder of the polarograph. In practice it was found that approximately 100 mg. dry wt., was a suitable amount. The cell contained 20 ml. of solution in which was dissolved approximately 300 μl. of oxygen. This quantity of oxygen was taken up by the slices in 5–10 min. However, with this rate of uptake the slope of the current–time curve was too low to permit the satisfactory use of the differential circuit to demonstrate breaks in the respiration rate–time curves. The trace we obtained on the polarograph showed a straight line initially and then the slope began to diminish progressively becoming zero at zero oxygen concentration. Duplicate measurements were made after fresh solution was run in. Provided that this was done slowly enough no slices were washed out. Duplicate values of C_s, the oxygen concentration at which the trace ceased to be linear, agreed with one another. From the slope of the linear portion the respiration rate could be determined. After sufficient replicate measurements had been made the slices were washed out of the cell with a solution of known oxygen concentration and a calibration point was obtained on the recorder of the polarograph. The slices were then dried for 2 hr. at 105°. From the dry weight and the slope of the linear portion of the trace the Q_{O_2} (μl. O_2/mg. dry wt./hr.) was calculated.

Table 1. *Relation of thickness of rat-liver slices to Q_{O_2} and the critical oxygen concentration*

C_s represents critical oxygen concentration, and d thickness. The slices marked (*a*) came from the same liver and similarly for those marked (*b*).

d (mm.)	Q_{O_2}	C_s (mM)
0·11 (*a*)	58	0·26
0·11 (*b*)	56	0·20
0·11	38	0·16
0·15	50	0·31
0·15	45	0·22
0·15	63	0·27
0·15	49	0·27
0·22	44	0·26
0·22	43	0·29
0·22	68	0·32
0·22	45	0·24
0·26	62	0·25
0·26 (*a*)	43	0·26
0·26 (*b*)	46	0·19
0·26	41	0·34
0·36	20	0·22
0·52	42	0·33
0·52	27	0·35
0·52	32	0·22
0·52	26	0·24

Table 2. *Relation of thickness of rat-kidney slices to Q_{O_2} and the critical oxygen concentration*

C_s represents the critical oxygen concentration for rat-kidney slices and d their thickness.

d (mm.)	Q_{O_2}	C_s (mM)
0·11	34	0·19
0·15	31	0·18
0·20	37	0·31
0·26	30	0·24
0·26	60	0·50
0·35	22	0·37
0·35	19	0·22
0·44	19	0·35
0·44	23	0·30
0·44	17	0·30
0·52	13	0·29
0·52	20	0·24
0·52	15	0·28
0·52	21	0·37

Table 3. *Relation of thickness of rat-heart slices to Q_{O_2} and the critical oxygen concentration*

C_s represents the critical oxygen concentration for rat-heart slices and d their thickness.

d (mm.)	Q_{O_2}	C_s (mM)
0·20	73	0·28
0·20	53	0·21
0·26	56	0·34
0·26	41	0·22
0·26	59	0·27
0·35	50	0·28
0·35	75	0·26
0·52	38	0·35
0·52	43	0·39

RESULTS

The results are shown in Tables 1–3. When the square of the thickness is plotted against C_s/Q_{O_2} the points should lie on a straight line, if Warburg's equation is valid. Fig. 1 shows the results obtained with rat-kidney slices. The correlation coefficients between these two variables were 0·661, 0·846 and 0·885 for liver, kidney and heart respectively. Each

of these values would occur less than once in 100 times by chance if there were no association.

These results are compatible with Warburg's equation and the regression lines and intercepts give measures of the diffusion coefficients and the critical oxygen concentrations of the terminal oxidase. The diffusion coefficients and critical oxygen concentrations are shown in Table 4.

In Warburg's equation the oxygen consumption and the concentration of oxygen are expressed in the units given in the introduction. We have expressed our experimental results for oxygen consumption in Q_{O_2} and oxygen concentration in moles/l.

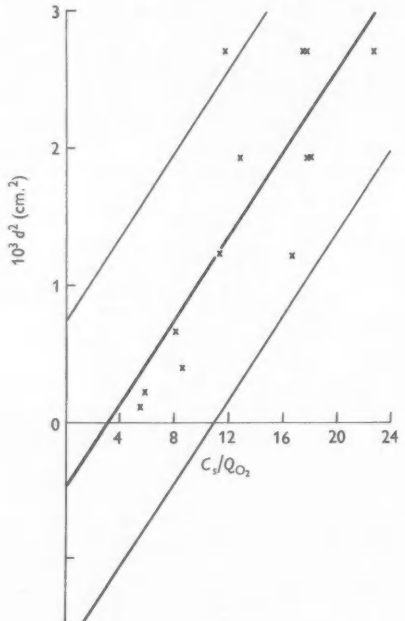

Fig. 1. A plot of the square of the thickness of rat-kidney slices against the ratio of the critical oxygen concentration to the Q_{O_2}. The middle line is the regression line and the others show the 95% confidence limits of the intercept.

DISCUSSION

The diffusion coefficients given in Table 4 are much higher than those found by Krogh (1919) and the critical oxygen concentrations are higher than those found in individual cells (Longmuir, 1957). These high figures suggest two things. First, even very thin slices only two or three cells thick have a critical oxygen concentration that is much higher than that of individual cells. Secondly, an increase in the thickness of a slice has a very small effect on its critical oxygen concentration, so it appears that oxygen can permeate rather freely.

It seems unlikely that the high concentration of succinate we used could have had an appreciable effect since, in the absence of any substrate, results of the same order were obtained. The C_e values were lower in the absence of succinate but approximately in proportion to the decrease in Q_{O_2}. Both sets of values were approximately halved. We have not quoted any figures obtained without added substrate as it was impossible to obtain exact replicates owing to the continued slow fall in the Q_{O_2}.

If the C_c truly is high, although this is not established since the standard error is so large, then cells in slices may contain a terminal oxidase in addition to cytochrome oxidase. Laws & Stickland (1956) have shown that liver cells have a greater respiratory activity than that due to the mitochondria they contain.

Since the figures we have obtained for the diffusion coefficient of oxygen through respiring tissues are higher than those found for non-respiring tissues the explanation must lie in some property associated with respiration. Some biological systems show a rhythmic ebb and flow of protoplasm (Seifriz, 1937). Such a movement in tissues might be enough to transport oxygen actively. It is well known that in the measurement of diffusion coefficients very slight vibration can increase enormously the apparent value. It seems likely that the mitochondria which appear to imbibe water continuously while they are actively extruding it may set up considerable microturbulence, particularly if they take up water over their whole surface and extrude it at certain 'spots'.

Seifriz & Epstein (1941) have shown that protoplasmic streaming can be stopped by a number of agents, such as mechanical violence, carbon dioxide and anaesthetics. In preliminary experiments we

Table 4. *Diffusion coefficients of oxygen through the three tissues and the critical oxygen concentration of the terminal oxidase calculated from the slopes and intercepts of the regression lines* (d^2 on C_e/Q_{O_2})

Organ	10^{-4} × Slope	10^{-4} × S.E. of slope	$10^4 D_{O_2}$ (Krogh's units)	Intercept	S.E. of intercept	C_c (mM)
Kidney	1·52	0·29	0·63	−0·048	0·120	0·093
Liver	3·92	0·88	1·64	−0·140	0·141	0·168
Heart	3·74	0·74	1·54	−0·093	0·108	0·126

have found that carbon dioxide increases the C_s value by up to 50%. We have not yet sufficient results to publish but they do lend support to the concept of an active intracellular transport of oxygen.

SUMMARY

1. The validity of Warburg's equation for diffusion of oxygen through tissue slices is discussed.

2. A method of studying the respiration of tissue slices is described which can be used to measure both their Q_{O_2} and their critical or limiting oxygen concentration.

3. The results obtained with this method support the validity of Warburg's equation.

4. The equation is used to calculate values for the diffusion coefficient of oxygen through respiring liver, heart and kidney slices, and values for the minimum concentration of oxygen necessary to saturate a terminal oxidase in those tissues.

5. Unexpectedly high values for the diffusion coefficients and possibly for the critical oxygen concentration of the terminal oxidase were obtained. Their significance is discussed.

We are indebted to Professor A. Haddow, F.R.S., of the Chester Beatty Research Institute for the supply of rats and to Dr P. Armitage of the School of Hygiene and Tropical Medicine for advice and assistance with the statistical examination of the results.

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Properties of L-α-Glycerophosphate Oxidase and its Role in the Respiration of *Trypanosoma rhodesiense*

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An aspect of host–parasite relationships of significance in chemotherapy concerns the biochemical differences which exist between pathogenic trypanosomes and the tissues of their vertebrate and invertebrate hosts. One important difference is that the blood-stream forms of many trypanosomes do not contain detectable cytochrome pigments (Brand, 1951; Ryley, 1956; Fulton & Spooner, 1959). Despite this, such parasites are notable for their high rates of respiration; for example, it has been calculated that the Q_{O_2} (glucose) of the African trypanosome, *Trypanosoma rhodesiense*, is about 50 times that of the tissues of its mammalian host (Brand, 1951).

The present work deals with hydrogen-transport systems present in a strain of the human parasite, *T. rhodesiense*, which has been maintained by syringe-passage in rats for many years. As far as is known the metabolism of this strain does not differ from that of the parasite freshly isolated from a human patient (Grant & Fulton, 1957; Fulton & Spooner, 1959). The results are mainly concerned with the properties of a L-α-glycerophosphate oxidase whose reaction with oxygen does not appear to be mediated either by pyridine nucleotides or a cytochrome system. The high activity and substrate specificity of this oxidase indicates that it can account for most of the respiration of the intact trypanosome *in vitro*.

A brief account of some of these results has already been presented (Grant & Sargent, 1959).

EXPERIMENTAL

Organism. The 'Liverpool' strain of *T. rhodesiense*, its maintenance in rats and the isolation of the parasite from infected rat blood have been described previously (Grant & Fulton, 1957).

Preparation of cell-free extracts. All procedures were carried out at 2°. A suspension of trypanosomes in iso-osmotic phosphate-buffered medium (Krebs & Eggleston, 1940) containing 0·2% of glucose was centrifuged at 1000 *g* for 10 min. After removal of the supernatant, the sedimented material was used to obtain cell-free preparations as described below.

The sedimented parasites (1 vol.) were suspended in water (4 vol.) with the aid of an all-glass homogenizer of the Potter–Elvehjem type (Loughborough Glass Co., Loughborough). After standing for 30 min. the suspension was centrifuged at 1000 *g* for 10 min. The supernatant, which was optically clear, was removed and the residue, which contained about 90% of the total N originally present in the starting material, was resuspended in water (6·5 vol.) and centrifuged at 1000 *g* for 10 min. The supernatant was removed and the residue resuspended in water as before. This final suspension contained about 1 mg. of N/ml. and will be referred to as the standard 'water-lysed' preparation.

The sedimented parasites (1 vol.) were suspended in 0·25 M-sucrose (4 vol.) containing 5 mM-ethylenediaminetetra-acetic acid (EDTA) previously adjusted to pH 7·4 by the addition of 0·1 N-NaOH. The suspension was then exposed to ultrasonic vibration for 60 sec. (MSE–Mullard ultrasonic disintegrator, stainless-steel vibrator probe with end-ratio 9:1; Measuring and Scientific Equipment Ltd., London). After being centrifuged at 1000 *g* for 10 min. the supernatant was removed and the slight residue discarded. This supernatant contained about 1·2 mg. of N/ml. and will be referred to as the 'ultrasonic' preparation.

Comparison of the oxygen utilization by cell-free preparations with that of intact parasites. In a typical experiment, 10 ml. portions of a trypanosome suspension were centrifuged at 1000 *g* for 10 min. and the supernatants discarded. The sedimented parasites in each tube (about 0·10 ml.) were then resuspended after the addition of one of the following: (*a*) phosphate-buffered medium (14 ml.) containing plasma albumin (140 mg.) and glucose (154 μmoles); (*b*) the same solution except that glucose was replaced by glycerol (216 μmoles); (*c*) water (2 ml.). After standing for 30 min. the three suspensions were centrifuged at 1000 *g* for 10 min. and the supernatants discarded. The 'water-lysed' preparation was resuspended in phosphate-buffered medium (14 ml.) containing plasma albumin (140 mg.) and the sodium salt of L-α-glycerophosphate (224 μmoles). The intact trypanosomes were resuspended in fresh medium (*a*) or (*b*) as described above and the oxygen uptake of each suspension (3 ml. samples) was determined.

Oxygen uptake. Oxygen consumption was measured in a conventional Warburg apparatus at 37° in air. The fluid volume added to each flask was 2·9 ml., except in the one experiment mentioned above. In all cases flasks were temperature-equilibrated for 15 min. before the start of the experiment. Except where otherwise noted, the reaction was started by tipping the substrate from the side arm. Cell-free extracts did not produce detectable CO_2 and, accordingly, KOH was not added to the centre well in these experiments.

Analytical methods. Total N was determined by the micro-Kjeldahl method, the NH_3 being distilled into a boric acid solution containing methyl red–methylene blue indicator.

Total phosphorus was determined as orthophosphate, after digestion with perchloric acid (sp.gr. 1·70) at 160°, by the method of Berenblum & Chain (1938) with the micro-modification of Long (1943).

α-Glycerophosphate (α-GP) was determined as orthophosphate after periodate oxidation followed by acid hydrolysis (Burmaster, 1946).

Total triose phosphate was determined as alkali-labile phosphate (Meyerhof & Lohmann, 1934).

In one group of experiments (Table 3), triose phosphate and α-GP were determined in the same sample. The reaction mixture was deproteinized by the addition of trichloroacetic acid to a final concentration of 5% (w/v) and the filtrate was extracted four times with an equal volume of freshly distilled diethyl ether to remove trichloroacetic acid. Triose phosphate was determined as the increase in orthophosphate observed in the residual solution after treatment with N-NaOH at 23° (alkali-labile phosphate). Under these conditions, it is possible that β-glycerophosphate (β-GP) was formed from the α-isomer originally present (cf. Long & Maguire, 1953), so that the total glycerophosphate in this solution was determined as the further increase in orthophosphate obtained by treatment of the acidified solution with periodic acid at 100° (Burmaster, 1946).

Individual triose phosphate esters were identified by enzymic methods with glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase. The procedure was essentially that described by Racker (1957)

Extinction changes in reactions involving diphosphopyridine nucleotide (DPN^+) were studied in a 1 cm.^{2} quartz cell at 340 mμ in the Hilger Uvispek spectrophotometer.

Materials. L-α-GP was synthesized as described by Baer (1952) except that quinoline present during the phosphorylation of *iso*propylidene-D-glycerol had to be removed by extraction with diethyl ether before the barium salt of L-α-GP would crystallize from hot water. DL-α-GP was synthesized as described by King & Pyman (1914) and isolated as the barium salt. The sodium salts of these compounds were used in all experiments and were obtained by the addition of a slight excess of sodium sulphate to a solution of the barium salt. After removal of the precipitate by centrifuging, the supernatant was neutralized and stored at −20° for not longer than 1 month before use. In these solutions, 93–96% of the total phosphate present could be accounted for as α-GP.

Triose phosphate isomerase was prepared from rat muscle as described by Beisenherz (1957).

The following materials were obtained commercially: pyridine nucleotides [DPN^+, reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN^+), reduced triphosphopyridine nucleotide (TPNH)], 2-amino-2-hydroxymethylpropane-1:3-diol (tris, recrystallized), mammalian cytochrome *c*, crystalline catalase, crystalline glyceraldehyde 3-phosphate dehydrogenase, from Boehringer und Söhne, Mannheim, Germany; adenosine di- and tri-phosphate (ADP, ATP) from the Sigma Chemical Co., St Louis, Mo., U.S.A.; bovine-plasma albumin fraction V from The Armour Laboratories, Sussex; Amytal (the sodium salt of 5-ethyl-5-*iso*amylbarbituric acid) from Eli Lilly and Co. Ltd., Basingstoke; mapharside (3-amino-4-hydroxyphenylarsenoxide hydrochloride) from Parke Davis and Co. Ltd., London.

The addition compound formed with dioxan and DL-glyceraldehyde-1-bromo-3-phosphoric acid, and the *cyclo*hexylamine salt of the dimethyl ketal of dihydroxyacetone

phosphate were obtained from the Nutritional Biochemical Corp., Calif., U.S.A. DL-Glyceraldehyde 3-phosphate was obtained by water hydrolysis of the former compound (Lardy, 1957) and from the latter dihydroxyacetone phosphate was prepared as described by Ballou & Fischer (1956). Crystalline yeast hexokinase and DL-*iso*citric acid lactone were obtained from L. Light and Co. Ltd., London. DL-*iso*Citric acid was prepared from the lactone as described by Deutsch & Phillips (1957).

Antimycin A was a gift from Dr E. F. Hartree and 2-heptyl-4-hydroxyquinoline *N*-oxide a gift from Dr J. W. Lightbown.

RESULTS

Cell-free preparations obtained from *T. rhodesiense* had a very limited ability to oxidize glucose, intermediates of the tricarboxylic acid cycle and substrate amounts of DPNH and TPNH. In contrast α-GP was rapidly oxidized at a constant rate over a period of 20–25 min., under the conditions given in Table 1. The racemic form was oxidized at half the rate of the L-isomer and in subsequent experiments it was shown that the latter is completely oxidized to dihydroxyacetone phosphate (Table 3 and Fig. 3). Neither glycerol nor β-GP was oxidized to an appreciable extent so that the preparation contains an α-GP oxidase which is stereochemically specific for the L-isomer.

Various means of disrupting the trypanosomes were tried, including the use of ultrasonic vibration, crushing in a press at −20° (Hughes, 1951) and freeze-drying, but the most active preparation was the particulate material obtained after lysis of the parasites in water. For this reason the 'water-lysed' preparation was subsequently used to determine some properties of the oxidase system, and was freshly prepared for each experiment, as some 70% of the original activity was lost after 24 hr. at 2°.

Kinetic aspects of the L-α-*glycerophosphate oxidase.* The effects of pH, enzyme and substrate concentration have been briefly investigated. These results are of interest not only as they help to characterize the system but also because they define the optimum conditions for the assay procedure.

To ensure that the pH of the medium was not affected by the buffering action of L-α-GP at neutral pH values, a high ratio of buffer to substrate was used and at the end of each experiment no appreciable change in pH had occurred, as determined by the glass electrode. It can be seen (Fig. 1) that in phosphate buffer there is an optimum activity at about pH 7·4, an activity of the same order being also found in tris buffer at this pH. For this reason, subsequent assays of enzyme activity were carried out in one or other of these buffers at pH 7·4, although it may be noted that the activity in tris buffer is slightly greater at higher pH values, with an optimum at about pH 8.

The activity of the enzyme with increasing substrate concentration is shown in Fig. 2. From these results the K_m was calculated from the reciprocal-plot method of Lineweaver & Burk (1934) to be 1·3 mM.

Oxidase activity under the experimental conditions given in Table 1 was found to be proportional to enzyme concentration up to 0·6 mg. of trypanosome N/flask.

Influence of various substances on the activity of L-α-*glycerophosphate oxidase. T. rhodesiense* contains no detectable catalase (Fulton & Spooner, 1956) and the addition of catalase had no effect on

Table 1. *Effect of various substances on the uptake of oxygen by cell-free preparation of* Trypanosoma rhodesiense

Each flask contained cell-free preparation (0·5 ml., approx. 0·5 mg. of N), tris buffer, pH 7·4 (0·14 M), and the additions as stated below, in a total volume of 2·9 ml. Oxygen uptakes were measured for 45 min., except with α-GP, when readings were discontinued after 25 min. —, Signifies value not determined.

Additions	Final concn. (mM)	Uptake of O_2 (μl./min./mg. of N) Standard 'water-lysed' preparation	Uptake of O_2 (μl./min./mg. of N) 'Ultrasonic' preparation
Nil	—	0	0
DL-α-GP	5·5	3·7	—
L-α-GP	5·5	7·6	7·0
L-α-GP and DPN+	5·5	7·1	—
Glycerol and ATP	6·2	0·9	—
DPNH or TPNH	7·6	0·5	0·5
Glycerol	6·2		
Glucose, ATP, and DPN+	6·9		
Fructose 1:6-diphosphate	5·5	<0·5	<0·5
Pyruvate	7·2		
Succinate	6·2		
DL-*iso*Citrate	9·0		

the activity of the enzyme (Table 2). Hydrogen peroxide, which is highly toxic to the enzyme, is thus unlikely to be a product, a view supported by the stoicheiometry of the reaction (Table 3).

The oxidase was markedly affected by *p*-chloromercuribenzoate, hydrogen peroxide and the trypanocidal drug, mapharside, which would indicate that one or more free thiol groups are essential for enzyme activity. In contrast, neither iodoacetic acid nor iodoacetamide was effective, although both of these compounds inhibit the oxygen uptake of intact trypanosomes (Brand, Tobie & Mehlman, 1950). This apparent anomaly will be referred to subsequently.

The oxidase was not significantly affected by compounds which normally inhibit the cytochrome systems of mammalian tissues (cf. Chance, 1957), an observation in agreement with the fact that *T. rhodesiense* does not contain detectable cytochrome pigments. The effect of azide was tested at pH 6·5

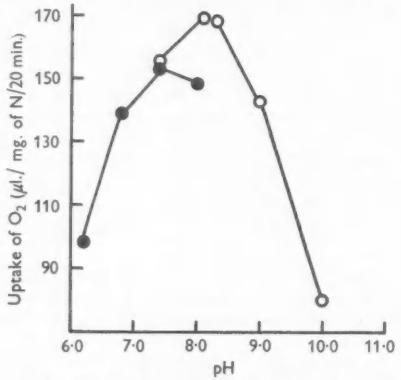

Fig. 1. Effect of pH on the activity of L-α-GP oxidase. Each flask contained the standard 'water-lysed' preparation (0·5 ml., approx. 0·5 mg. of N), buffer (0·14 M) and L-α-GP (5·5 mM), in a total volume of 2·9 ml. pH of flask contents was determined by glass electrode at about 20°. ●, Phosphate buffer; ○, tris buffer.

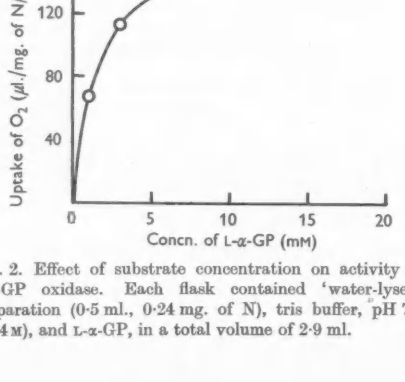

Fig. 2. Effect of substrate concentration on activity of L-α-GP oxidase. Each flask contained 'water-lysed' preparation (0·5 ml., 0·24 mg. of N), tris buffer, pH 7·4 (0·14 M), and L-α-GP, in a total volume of 2·9 ml.

Table 2. *Effect of various substances on the activity of* L-α-*glycerophosphate oxidase*

Each flask contained 'water-lysed' preparation (0·5 ml., approx., 0·5 mg. of N), tris buffer, pH 7·4 (0·14 M), and L-α-GP (5·5 mM) and the substance under test (0·1 ml.) in a total volume of 2·9 ml. All substances were in aqueous solution, except *p*-chloromercuribenzoate and 2-heptyl-4-hydroxyquinoline *N*-oxide, which were dissolved in 0·05 N-NaOH, and antimycin A, which was dissolved in aqueous 50 % (v/v) ethanol. After equilibration for 15 min. the reaction was started by the addition of L-α-GP from the side arm. Oxidase activities are expressed as µl. of O_2/0·5 mg. of N in 25 min.

Additions	Final concn. (mM)	Oxidase activity
Nil	0	100
Hydrogen peroxide	1·0	0
p-Chloromercuribenzoate	0·1	2
N-Ethyl maleimide	1·0	8
Mapharside	0·1	41
Iodoacetate*	1·0	100
Iodoacetamide*	1·0	96
NaCN	10	96
NaN_3†	10	94
NaF	1·0	100
Amytal	1·0	95
Antimycin A	5 µg./flask	98
2-Heptyl-4-hydroxyquinoline *N*-oxide	10 µg./flask	93
Cytochrome *c*	1 mg./flask	100
Catalase	0·0005	100
EDTA	1·0	110
Plasma albumin	29 mg./flask	115

* Either 15 or 30 min. equilibration with enzyme before assay.

† Assayed in both tris buffer, pH 7·4, and phosphate buffer, pH 6·5.

Table 3. *Quantitative relationship between substrate and product of the* L-α-*glycerophosphate-oxidase reaction*

Each flask contained water-lysed preparation (0·5 ml., 0·3 mg. of N in Expts. 1 and 2, and 0·5 mg. of N in Expt. 3), tris buffer, pH 7·4 (0·14 M), KCN (10 mM) and L-α-GP (15·5 µmoles), in a total volume of 2·9 ml. In Expts. 1 and 2 the reaction was stopped, after 18 min., by rapid cooling to 0° with the simultaneous addition of 0·5 ml. of 30 % (w/v) trichloroacetic acid. In Expt. 3 the reaction was stopped in a similar manner after 25 min. —, Signifies value not determined.

Expt. no.	L-α-GP used (µmoles)	Uptake of O_2 (µg.atoms)	Triose phosphate formed (µmoles)
1	8·6	9·0	8·2
2	7·9	8·5	7·7
3	—	14·6	14·2

as well as at pH 7·4 since it has been stated (Keilin, 1936) that azide does not significantly affect cytochrome systems at neutral or slightly alkaline pH values.

Stoicheiometric relationships of the L-α-*glycerophosphate-oxidase reaction.* To determine the quantitative relationship between reactants and products, enzyme and enzyme–substrate suspensions were analysed at the beginning and end of each experiment. The net decrease in the amount of L-α-GP present and the corresponding increase in triose phosphate for each experiment are given in Table 3. It can be seen that when nearly all or only half of the substrate has been utilized the results are consistent with the reaction:

$$\text{L-α-GP} + \tfrac{1}{2}O_2 \rightarrow \text{triose phosphate} + H_2O. \quad (1)$$

The equilibrium condition of the reaction clearly favours the oxidation of L-α-GP, although cyanide which was added to stabilize triose phosphate as the cyanohydrin may have affected the equilibrium. This latter possibility is, however, unlikely, since the complete oxidation of L-α-GP in accordance with reaction (1) has been consistently observed in the absence of cyanide (Fig. 5).

It was found (Fig. 3) that dihydroxyacetone phosphate was the only triose phosphate ester present in the protein-free reaction mixture at the end of each experiment. The oxidase preparation itself did not contain detectable triose phosphate isomerase since it did not catalyse the reduction of DPN^+ on the addition of glyceraldehyde 3-phosphate dehydrogenase and synthetic dihydroxyacetone phosphate, as judged from the extinction of the solution at 340 mμ. These results show that the product of the oxidase reaction is dihydroxyacetone phosphate and not glyceraldehyde 3-phosphate.

In order to determine whether oxidation of L-α-GP was coupled to phosphorylation, the following additions were made to each of the reaction mixtures containing L-α-GP that were described in Table 1: glucose (1·7 mM), ATP (1·0 mM), hexokinase (152 units, Berger, Slein, Colowick & Cori, 1946), magnesium chloride (3·4 mM) and orthophosphate (9·5 mM). In no case, however, was there any significant decrease in the concentration of orthophosphate during the experimental period.

Enzyme and enzyme sequences of the Embden–Meyerhof glycolytic pathway present in T. rhodesiense. The supernatant obtained by lysis of *T. rhodesiense* with water gave no significant oxygen uptake with L-α-GP or substrate amounts of DPNH. Nevertheless, added DPNH was rapidly oxidized, as judged by the decrease in extinction of the solution at 340 mμ (Fig. 4). This effect was abolished by dialysis, but the activity could be restored by the addition of dihydroxyacetone phosphate, indicating the presence of a soluble

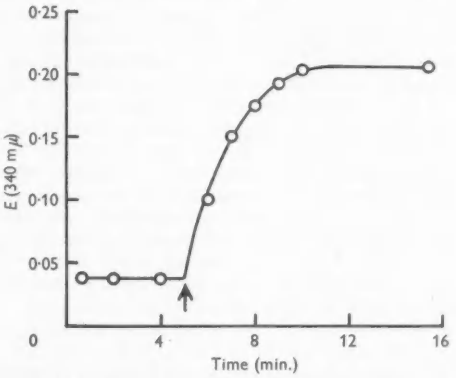

Fig. 3. Dihydroxyacetone phosphate as the product of L-α-GP-oxidase reaction. Cuvettes contained protein-free incubation medium from experiments described in Table 3, pyrophosphate buffer, pH 8·6 (120 μmoles), sodium arsenate (15 μmoles), DPN^+ (0·7 μmole) and crystalline glyceraldehyde 3-phosphate dehydrogenase (0·1 mg.). Triose phosphate isomerase (2 units, Beisenherz, 1957) was added as shown by the arrow. Total volume, 2·9 ml. Control cuvette: protein-free incubation medium omitted.

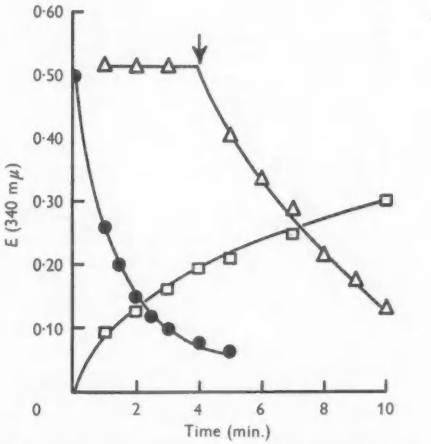

Fig. 4. L-α-GP-dehydrogenase activity in cell-free extracts of *T. rhodesiense*. Cuvettes contained 2·7 ml. of reaction mixture. ●, Phosphate buffer, pH 7·4 (200 μmoles), $MgCl_2$ (10 μmoles) and DPNH (0·3 μmole). The reaction was started by addition of the supernatant from a 'water-lysed' preparation (0·2 ml., 0·1 mg. of N). △, Mixture as given above except that the supernatant had been dialysed. Dihydroxyacetone phosphate (1 μmole) was added at the time shown by the arrow. □, Hydrazine–HCl buffer, pH 9·2 (1 m-mole), $MgCl_2$ (10 μmoles), L-α-GP (16 μmoles), DPN^+ (0·5 μmole) and dialysed supernatant. Control cuvette: pyridine nucleotide omitted.

DPN-linked L-α-GP dehydrogenase. Reduction of DPN^+ in the presence of L-α-GP could be achieved only at a higher pH and in the presence of hydrazine to trap the triose phosphate. The equilibrium of the reaction thus favours the formation of L-α-GP from dihydroxyacetone phosphate (cf. Baranowski, 1949). This enzyme, like the particulate L-α-GP oxidase, was inhibited by *p*-chloromercuribenzoate (0·1 mM), but not by iodoacetate (1·0 mM). Aldolase and triose phosphate isomerase were shown to be present in the supernatant by the oxidation of DPNH in the presence of fructose 1:6-diphosphate or glyceraldehyde 3-phosphate. Triose phosphate isomerase was insensitive to iodoacetate (1·0 mM). Glyceraldehyde 3-phosphate dehydrogenase could not be detected by the usual spectrophotometric method, presumably because DPNH formed in the reaction was immediately reoxidized by the very active DPN-linked L-α-GP dehydrogenase. This explanation is supported by the following experiment, the results of which are given below.

Trypanosomes were lysed in water and the complete cell-free suspension was dialysed. The non-diffusible suspension utilized added L-α-GP with an uptake of 1 g.atom of oxygen/mole of L-α-GP, the oxygen uptake being doubled when DPN^+ and L-α-GP were added together (Fig. 5). This latter result is in contrast with the lack of stimulation of added DPN^+ on L-α-GP oxidation by the washed particulate preparation (Table 1). It is thus probable that triose phosphate formed in the oxidation of L-α-GP (reaction 1) reduces DPN^+ in the glyceraldehyde 3-phosphate-dehydrogenase reaction (reaction 6 below) and DPNH thus formed is reoxidized by the soluble L-α-GP dehydrogenase with the formation of L-α-GP from triose phosphate (reaction 3 below). The overall result of these coupled reactions would be that found experimentally, i.e. 1 mole of oxygen consumed/mole of L-α-GP oxidized. This interpretation is supported by the finding that iodoacetate decreases the uptake of oxygen to approximately that found for the oxidase reaction alone (Fig. 5). In the present results the triose phosphate isomerase and the L-α-GP oxidase and dehydrogenase of *T. rhodesiense* have all been shown to be insensitive to iodoacetate, whereas it is known that glyceraldehyde 3-phosphate dehydrogenases of mammalian tissues, yeasts and trypanosomes closely related to *T. rhodesiense* (Marshall, 1948; Harvey, 1949) are all strongly inhibited by iodoacetate.

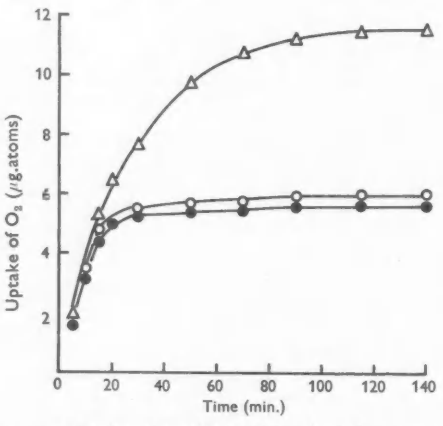

Fig. 5. Oxidation of L-α-GP by dialysed cell-free suspension of *T. rhodesiense*. Trypanosomes were lysed in water and the complete suspension was dialysed for 6 hr. at 2° against a mixture of 50 vol. of phosphate-buffered medium (Krebs & Eggleston, 1940) and 450 vol. of water. ●, Flasks contained non-diffusible suspension (1 mg. of N), phosphate-buffered medium, pH 7·4 (2 ml.), plasma albumin (29 mg.), reduced glutathione (0·15 μmole) and L-α-GP (5·6 μmoles), in a total volume of 2·9 ml. △, System, as described above, DPN^+ (1 μmole) and NaF (2·9 μmoles); ○, as described above, DPN^+ (1 μmole) and iodoacetate (2·9 μmoles). Oxygen uptake was negligible on omission of L-α-GP.

Relationship between the activity of L-α-*glycerophosphate oxidase and the respiration of the intact trypanosome.* In a series of four experiments, the rate of oxygen uptake of intact trypanosomes has been directly compared with the activity of the oxidase isolated from an equivalent number of parasites. Maximum initial rates were obtained by the use of substrate concentrations so high that substrates did not become rate-limiting over the experimental period (see Fig. 2). A typical result is given in Fig. 6, where it can be seen that the oxygen uptake of the preparation was about 60 and 110% of that of the intact trypanosome utilizing glycerol and glucose respectively. This would suggest that the rate of conversion of glycerol into L-α-GP is faster than that of glucose. Indeed under these conditions only trace amounts of L-α-GP were detectable in the trypanosome when glucose was used as substrate. In contrast, the parasite respiring in glycerol contained 2·1 μmoles of α-GP/mg. of trypanosome N. On the assumption that there is only one hydrogen-transport system in *T. rhodesiense*, the oxidase must be a rate-limiting step in the metabolism of glycerol and must function at near its maximum activity. It is thus probable that the correspondence between the respiration rate of the intact trypanosome metabolizing glucose and the oxidase activity is fortuitous and that the isolated enzyme system contains only about 60% of the oxidase activity present in the intact trypanosome.

The isolated oxidase system was found to be

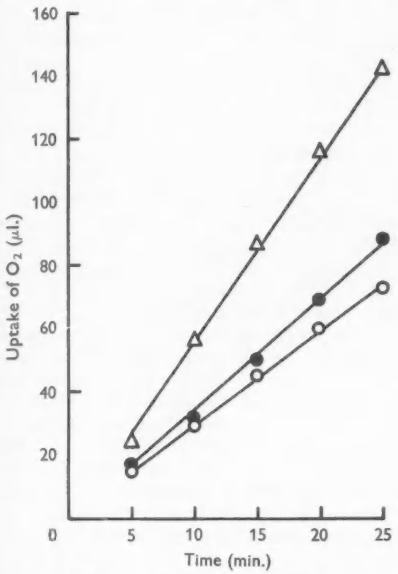

Fig. 6. Rate of oxygen uptake by a suspension of *T. rhodesiense* compared with that by the L-α-GP oxidase isolated from the same suspension. Details are given in the Experimental section. ●, Oxidase preparation (isolated from intact trypanosome suspension containing 0·29 mg. of N) with L-α-GP (16 mM) as substrate. ○, Intact organism (0·29 mg. of N) with glucose (11 mM) as substrate. △, Intact organism (0·29 mg. of N) with glycerol (15·4 mM) as substrate.

very unstable in water and accordingly attempts were made to obtain enhanced enzyme activity by decreasing the standard time of water-lysis of the intact parasite. Unfortunately, water-lysis for periods of 20 min. or less resulted in the presence of intact trypanosomes in the preparation and this approach was accordingly abandoned in the present experiments.

DISCUSSION

Many tissues contain both a cytoplasmic DPN-linked α-GP dehydrogenase and a particulate α-GP-oxidase system which reacts directly with oxygen. These enzymes have been found in mammalian tissue (Green, 1936; Green, Needham & Dewan, 1937; Young & Pace, 1958), in insect muscle (Bücher & Klingenberg, 1958; Estabrook & Sacktor, 1958*a*) and in the midgut of the silkworm (Ito & Horie, 1959). The present results show that enzymes of both types are present in the protozoon *T. rhodesiense*.

The L-α-GP oxidase studied in these experiments differed from the oxidase present in mammalian-brain mitochondria (Sacktor, Packer & Estabrook, 1959; Ringler & Singer, 1959) and insect-muscle sarcosomes (Estabrook & Sacktor, 1958*a*, *b*) in that its reaction with oxygen was not mediated by a cytochrome system and its activity was unaffected by either EDTA or sodium fluoride. Nevertheless, the oxidase was associated with the particulate material of the cell, but no attempt was made to determine its distribution in subcellular particles. There is no evidence that trypanosomes contain mitochondria (Anderson, Saxe & Beams, 1956), although such structural features are present in larger protozoa (Wolken & Palade, 1953).

Apart from the sensitivity to either alkylating or mercaptide-forming reagents, which would suggest that one or more thiol groups are essential for enzyme activity, virtually nothing is known about the mechanism of hydrogen transport in the L-α-GP oxidase. Pyridine nucleotides do not appear to be involved since neither DPN^+ nor TPN^+ enhances oxidase activity. The possibility that the system is already saturated with firmly bound coenzyme cannot be excluded, but it may be noted that the enzyme was insensitive to Amytal, a compound known to inhibit the reaction of some DPN-linked dehydrogenases with flavoproteins of mitochondria and sarcosomes (Chance & Baltscheffsky, 1958). Experiments by Ryley (1956) and Fulton & Spooner (1959) would suggest that cell-free preparations of *T. rhodesiense* contain a cytochrome *c* oxidase. However, the intact parasite does not contain detectable cytochrome pigments and its respiration is insensitive to cyanide, azide and carbon monoxide (Fulton & Spooner, 1959), so that the significance of these experiments to the mechanism of hydrogen transport is obscure at the present moment.

The respiration and motility of the trypanosome isolated from rat blood is entirely dependent on an extracellular supply of glucose or other substrate such as glycerol. Under these conditions, glucose is metabolized by the Embden–Meyerhof glycolytic pathway to pyruvate and a trace of glycerol (Grant & Fulton, 1957), about 1 mole of oxygen being taken up/mole of glucose utilized (Ryley, 1956). Pyruvate is not metabolized further (Grant & Fulton, 1957) and it has been assumed that DPNH formed in the glyceraldehyde 3-phosphate-dehydrogenase reaction was reoxidized by direct reaction with a hydrogen-transport system. The finding that undialysed crushed-cell suspensions of *T. rhodesiense* had a very low DPNH-oxidase activity (Fulton & Spooner, 1959) is in agreement with the present results, in which a variety of possible hydrogen donors, including DPNH, were utilized at about 5% of the rate found for L-α-GP. These results, together with the fact that the activity of the isolated oxidase can account for the

major part of the respiration of the intact trypanosome, would indicate that this enzyme system must play an important role in the oxidative metabolism of the organism. On the evidence available, it is therefore suggested that the reactions catalysed by the particulate oxidase (2) and the cytoplasmic DPN-linked L-α-GP dehydrogenase (3) are coupled and constitute a hydrogen carrier system for the reoxidation of DPNH.

$$\text{L-α-GP} + \tfrac{1}{2}O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O \quad (2)$$

$$\text{Dihydroxyacetone phosphate} + \text{DPNH} + H^+ \rightleftharpoons \text{L-α-GP} + \text{DPN}^+ \quad (3)$$

Sum: $$\text{DPNH} + H^+ + \tfrac{1}{2}O_2 \rightarrow \text{DPN}^+ + H_2O.$$

This scheme is feasible since the equilibrium condition of reaction (2) results in essentially complete oxidation of L-α-GP (Table 3). Moreover, the reaction catalysed by the dehydrogenase favours L-α-GP formation (Fig. 4), a result in agreement with the equilibrium constant of the reaction $K_e = 5{\cdot}5 \times 10^{-12}$ M at 25° and at an ionic strength of about 0·03 (Burton & Wilson, 1953). A similar scheme has been suggested by Bücher & Klingenberg (1958) and Estabrook & Sacktor (1958*a*) to account for the respiration of insect flight muscle under certain conditions. In the present experiments, however, the coupling of these reactions has been demonstrated in a cell-free suspension of *T. rhodesiense*, the constitutive glyceraldehyde 3-phosphate dehydrogenase being used to generate reducing equivalents of DPNH. In this experiment (Fig. 5), about 1 mole of oxygen was utilized/mole of L-α-GP added (reactions 4, 5 and 6).

This scheme can satisfactorily account for the oxidative catabolism of glucose or glycerol to pyruvate. The essential details of these reactions are illustrated in Fig. 7, where it may be noted that the oxidation of DPNH involves indirect hydrogen transfer to the respiratory chain via L-α-GP.

It has been repeatedly observed that the respiration of the blood-stream form of *T. rhodesiense* was extremely sensitive to alkylating and mercaptide-forming reagents (Brand *et. al* 1950; Ryley, 1956) and to oxidizing agents such as hydrogen peroxide (Fulton & Spooner, 1959). The present results indicate that these compounds, with the exception of iodoacetate, can act directly on the hydrogen-transport system. Iodoacetate has been shown to inhibit the glyceraldehyde 3-phosphate dehydrogenase of *T. rhodesiense* (Fig. 5) and of other closely related trypanosomes (Marshall, 1948; Harvey, 1949), so that inhibition of this enzyme could account for its effect on the respiration of the intact organism.

Further discussion on the results presented here may be more usefully undertaken when the components of the hydrogen-transport system have been identified and when more is known about the possible changes that may be brought about in the structure and function of the system during its isolation from the organism.

$$2\ \text{L-α-GP} + O_2 \rightarrow 2\ \text{triose phosphate} + 2H_2O \quad (4)$$

$$\text{Triose phosphate} + \text{DPNH} + H^+ \rightarrow \text{L-α-GP} + \text{DPN}^+ \quad (5)$$

$$\text{Triose phosphate} + \text{DPN}^+ + H_3PO_4 \rightarrow \text{1:3-diphosphoglyceric acid} + \text{DPNH} + H^+ \quad (6)$$

Sum: $$\text{L-α-GP} + O_2 \rightarrow \text{1:3-diphosphoglyceric acid} + 2H_2O$$

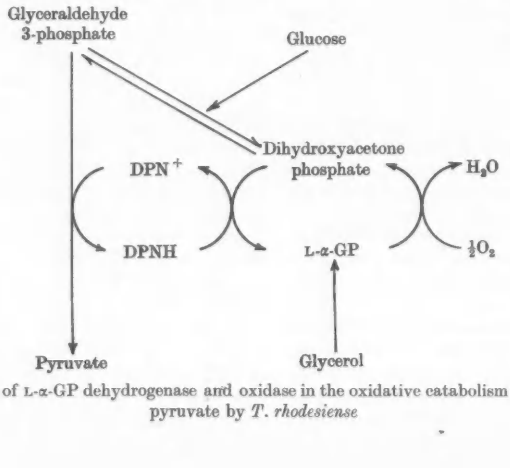

Fig. 7. Suggested role of L-α-GP dehydrogenase and oxidase in the oxidative catabolism of glucose or glycerol to pyruvate by *T. rhodesiense*

SUMMARY

1. Cell-free extracts of *Trypanosoma rhodesiense* have been found to contain a cytoplasmic L-α-glycerophosphate dehydrogenase and a particulate hydrogen-transport system whose rate of reaction with L-α-glycerophosphate was about 20 times that of other possible hydrogen donors such as reduced diphosphopyridine nucleotide or succinate.

2. The latter enzyme system has been termed L-α-glycerophosphate oxidase, as 1 g.atom of oxygen was utilized in the oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate.

3. The optimum activity of the oxidase in phosphate buffer and in 2-amino-2-hydroxymethylpropane-1:3-diol buffer was about pH 7·4 and pH 8·0 respectively. At pH 7·4 the K_m was calculated to be 1·3 mM.

4. The activity of the oxidase was inhibited by certain thiol reagents but not at all by compounds which inhibit systems containing cytochrome pigments. There was no evidence that pyridine nucleotides are involved in the oxidase reaction.

5. Under conditions where the substrate concentration was not a limiting factor over the experimental period, the rate of uptake of oxygen by the oxidase was about 60 and 110% of that found for the intact trypanosome respiring in either glycerol or glucose respectively.

6. A dialysed cell-free suspension of *Trypanosoma rhodesiense* utilized 1 g.atom of oxygen/mole of L-α-glycerophosphate added but this uptake of oxygen could be doubled when oxidized diphosphopyridine nucleotide was also added.

7. It is suggested that the reactions catalysed by the diphosphopyridine nucleotide-linked dehydrogenase and the oxidase are coupled and constitute a hydrogen-transport system for the oxidation of reduced diphosphopyridine nucleotide. The compatability of this suggestion with previous work on the oxidative catabolism of glucose by *Trypanosoma rhodesiense* has been discussed.

We are greatly indebted to Professor W. O. Kermack, F.R.S., for many helpful discussions during the course of this work. One of us (J.R.S.) wishes to thank the Carnegie Trust for a Scholarship.

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Manometric Assay and Properties of Pancreatic Cholesterol Esterase

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It has long been known that enzymically catalysed synthesis and hydrolysis of cholesterol esters occurs in the presence of certain tissues (Kondo, 1910*a*, *b*; Schultz, 1912; Cytronberg, 1912; Gardner & Lander, 1913; Mueller, 1916*a*, *b*; Porter, 1916; Nomura, 1924*a*, *b*; Shope, 1928; Nedswedski, 1935, 1936; Klein, 1939; Sperry, 1935; Sperry & Stoyanoff, 1937, 1938). The most extensive recent studies, initiated with the enzyme from pancreas, are those of Yamamoto, Goldstein & Treadwell (1949) and Swell & Treadwell (1950*a*), who stressed the importance of stable emulsions of cholesterol and cholesterol esters and described their preparation. By use of these mixtures numerous properties of the cholesterol esterase present in serum (Swell & Treadwell, 1950*b*) and in homogenates of various organs were studied (Swell, Byron & Treadwell, 1950; Byron, Wood & Treadwell, 1953; Swell, Dailey, Field & Treadwell, 1955). Hydrolysis of cholesteryl acetate catalysed by an enzyme present in the microsomal fraction of rat liver has been observed by Schotz, Rice & Alfin-Slater (1954). Some properties of purified pancreatic cholesterol esterase have been reported by Hernandez & Chaikoff (1957), who devised a rapid turbidimetric method for estimation of enzymic activity.

The present report describes some characteristics of the cholesterol esterase, which is found in extracts of acetone-dried pancreas, and defines the conditions required to demonstrate high catalytic activity. The data were obtained by use of a manometric procedure for estimation of synthesis or hydrolysis of cholesterol esters. Brief reports of some of the data have been made (Korzenovsky, Rust & Diller, 1955; Korzenovsky, Vesely & Diller, 1956).

METHODS

In this paper the expression colloidal substrate is used to describe a properly prepared colloidal system consisting of sterol or sterol ester with albumin, fatty acid and bile acid.

Preparation of colloidal substrate and composition of standard test system for synthesis of sterol esters. The substrate for esterification consisted of sterol, fatty acid (used as the lithium salt unless otherwise noted) and bile acid. Appropriate amounts of these compounds were placed in a flask containing approximately the requisite amount of water. To this was added an amount of ethyl ether equal to one-half of the volume of water. The flask was shaken until all components dissolved and only one phase was present. The ether was expelled by gentle stirring while warming on the water bath. The temperature was not allowed to rise above 50°. The substrate was adjusted to pH 6·5–6·6 with 0·1 M-H_2SO_4 and made up to volume. Ether was again added and the mixture was re-emulsified, by shaking, until it became single-phased. The ether was removed as above. Although the colloidal substrate for esterification may be prepared as described at virtually any concentration, so long as the ingredients are maintained in the proper molar ratio, the following concentrations have been found convenient to use in the standard substrate for synthesis of cholesteryl oleate: 0·04 M-cholesterol, 0·12 M-lithium oleate and 0·02 M-cholic acid; 0·8 ml. of this was used in every assay. The final concentrations in a volume of 2·2 ml. were: 0·014 M-$NaHCO_3$, 0·09 M-NaCl or 0·045 M-$(NH_4)_2SO_4$, 0·0145 M-cholesterol, 0·044 M-lithium oleate and 7·3 mM-cholic acid. To ensure rapid gas equilibration the total fluid volume did not exceed 2·2 ml. The complete reaction mixture after gassing with CO_2 is at pH 6·1–6·2.

Preparation of colloidal substrate and composition of standard test system for hydrolysis of sterol esters. The components of the substrate for hydrolysis, consisting of sterol ester, bile acid and lithium oleate, were placed in an appropriate amount of an aqueous 0·67% solution of crystalline bovine albumin. The mixture was shaken with a volume of ether equal to one-half of the amount of water until only one phase was present. After the ether was removed by gentle heat, the substrate was adjusted to pH 6·9–7·0 and made up to volume with the albumin solution. The remainder of the procedure is as outlined in the previous section. The standard substrate for hydrolysis consists of 0·07 M-cholesteryl oleate, 0·014 M-cholic acid and 0·035 M-lithium oleate emulsified in 0·67% albumin solution as described. The ratio of the first two compounds on a molar basis is 5:1. The concentrations of lithium oleate and albumin are not critical but appear to be minimal as given. The standard test system consists of the following ingredients, which must be placed in the reaction vessel in the order listed to preserve the dispersed character of the substrate: 0·8 ml. of the substrate for hydrolysis, water (in sufficient amount so that the final fluid volume is 2·2 ml.), $NaHCO_3$, $(NH_4)_2SO_4$ at pH 7·0, and enzyme. The final concentrations were 0·18 M-$NaHCO_3$, 0·72 M-$(NH_4)_2SO_4$, 0·025 M-cholesteryl oleate, 5·1 mM-cholic acid, 0·0127 M-lithium oleate and 0·24% albumin. After flushing with CO_2 the complete reaction mixture is at pH 6·9–7·0.

Although the substrates prepared as described do not deposit a sediment or change in any discernible way, even after several days of storage, only quantities sufficient for daily use were made at any one time. The mixtures should not be frozen.

Manometry. The reactions were conducted at 37° in single-side-arm Warburg flasks of approx. 16 ml. capacity. The enzyme solution was put in the side arm and all other ingredients in the main compartment unless otherwise indicated. After gassing with 100% CO_2 and temperature equilibration, the reaction was begun by tipping the enzyme into the main compartment. The first reading was made after 3–6 min., and the reaction was allowed to proceed for 30 min. more. Results are reported as μl. of CO_2. The absorption of gas is designated by a minus sign and is indicative of ester synthesis. Release of CO_2 occurs during ester hydrolysis and is indicated by a plus sign. Bicarbonate solutions were gassed with CO_2 for at least 30 min. before use.

Enzyme solution. Ox pancreas, obtained at slaughter, was chilled in ice immediately after removal from the carcass. Fat and connective tissue were trimmed away and the rest of the gland was cut into small pieces, but not minced. The pieces of pancreas were rapidly stirred with 10 times their volume of acetone at −15° and were left overnight at this temperature. The acetone was decanted and the solid material was passed through an ordinary meat grinder into a volume of acetone equal to the first used. The grinding operation and subsequent filtrations were done at −10° to −15°. The suspension was filtered on a Büchner filter without permitting the cake to dry. The moist cake was washed on the filter with fresh acetone, then with acetone–ether (1:1, v/v) and finally with ether in amounts equal to twice the volume of the filter cake. The residue was sucked dry and was spread on a clean surface to eliminate the ether. The dry powder has been kept at −15° for periods longer than 1 year without appreciable loss of activity. A solution of the enzyme was prepared by suspending 1 g. of powder in 10 ml. of 0·01 M-NaCl or water at 4°. The suspension was stirred at 4° for 30–60 min. and then was filtered through a hard-surface paper. The clear amber-coloured solution contained 15–20 mg. of protein/ml. as determined according to Weichselbaum (1946). It may be dialysed for 48 hr. against 0·01 M-NaCl or for 4–6 hr. against water without loss of activity. Almost all of the enzymically active protein present in the extract was precipitated upon increasing the concentration of $(NH_4)_2SO_4$ from 25 to 50% of saturation. The precipitate was dissolved in water and was dialysed for 4–6 hr. against water or for 24 hr. against 0·01 M-NaCl. The solution contained 5–7 mg. of protein/ml., indicating an approximately threefold concentration of the enzyme. About 0·3 ml. of either enzyme preparation was used in each Warburg flask. The crude extract and the fraction obtained by $(NH_4)_2SO_4$ precipitation were found equally satisfactory in the manometric procedure.

Chemicals. Sterols were obtained from commercial sources; they were recrystallized until m.p. values and X-ray-diffraction patterns agreed with published data. β-Sitosterol was of cotton seed origin. β-Norcholesterol was kindly supplied by Dr W. G. Dauben (University of California, Berkeley). Oleic acid was purified according to Hilditch (1947). The lithium salt was obtained by neutralizing the purified acid with LiOH followed by precipitation with acetone. The crystalline bile acids were a generous gift of Professor S. Bergström (Lund, Norway), to whom a debt of gratitude is expressed.

RESULTS

Estimation of cholesterol-esterase activity. The manometric estimation of cholesterol-esterase activity is based on the assumption that for each mole of ester which is synthesized or hydrolysed an equivalent of CO_2 gas is exchanged. The estimation of ester hydrolysis, by measurement of CO_2 gas released from the aqueous phase of a sodium bicarbonate–CO_2 buffer system, is a well-established analytical procedure. The reverse process, i.e. the measurement of the amount of CO_2 gas absorbed into the aqueous phase of a sodium bicarbonate–CO_2 buffer system to estimate ester synthesis, is not a method in common use. It is evident from Table 1 that, under the conditions specified, the manometric determination of synthesis or hydrolysis of cholesteryl oleate is in fair agreement with results obtained by the colorimetric procedure of Sperry & Webb (1950). Table 2 shows that the CO_2 gas absorbed into the aqueous phase appears as bicarbonate or a closely related compound. The

Table 1. *Comparison of manometric and colorimetric estimation of cholesterol-esterase activity*

The standard test systems for synthesis and hydrolysis of cholesteryl oleate were used except that the reactions were allowed to proceed for the times indicated. Cholesterol was determined by the method of Sperry & Webb (1950) on a sample of the reaction mixture used for manometric estimation. Synthesis of cholesteryl oleate is indicated by a decrease (−) of cholesterol or CO_2 and hydrolysis by an increase (+) of these compounds.

Method of estimation ...		Colorimetric (*A*)	Manometric (*B*)	
Reaction	Time of incubation (min.)	Cholesterol (μmoles)	CO_2 (μmoles)	*B*:*A*
Synthesis	30	−3·0	−3·3	1·10
	75	−7·3	−8·1	1·11
	165	−15·6	−13·9	0·89
Averages		−8·6	−8·4	1·03
Hydrolysis	30	+6·8	+6·0	0·88
	60	+8·5	+9·6	1·13
	110	+11·0	+12·1	1·11
Averages		+8·8	+9·2	1·04

amount of CO_2 gas absorbed during esterification is very nearly equal to the amount released upon addition of mineral acid. Table 3 shows that 42% of the radioactivity initially present as [4-^{14}C]-cholesterol is found in a compound that reacts with digitonin only after hydrolysis. Nearly the same percentage of the initial radioactivity is associated with a compound that follows the elution pattern of an authentic sample of cholesteryl oleate during isolation by silicic acid chromatography. The enzyme has been found useful for preparation of labelled sterol esters.

Table 2. *Conversion of carbon dioxide into bicarbonate during synthesis of cholesteryl oleate*

To accommodate the large change in gas volume a differential manometer of the Summerson type was used. The volumes of each side of the assembled manometer were nearly identical as judged by the volume of CO_2 released from a $NaHCO_3$ solution. The reaction was conducted in double-side-arm flasks. The main compartments of each flask contained an identical amount of the standard reaction mixture for synthesis of cholesteryl oleate. The enzyme was placed in one side arm and 0·2 ml. of N-H_2SO_4 in the other. The reaction was begun in flask 3 by tipping the enzyme into the main compartment. After 2 hr. of activity, acid was added from the second side arm and the contents of both side arms and the main compartment of flask 6 were mixed. The data are given as millimetres of change in manometer fluid.

	Reaction flask 3 (mm.)	Control flask 6 (mm.)	Total change (mm.)
After 2 hr. of activity	−132	+68	−200
After acidification	+232	+26	+206

Properties of cholesterol esterase

Influence of substrate composition and concentration upon rate of esterification. Dependence of the rate of cholesteryl oleate synthesis upon the concentration of cholesterol, oleate and cholic acid is illustrated in Figs. 1–3. These data were obtained with substrates in which the concentration of only one component at a time was varied from zero to the levels indicated on the graphs. However, it was not possible to prepare a stable emulsion of cholesterol and cholic acid in the complete absence of oleate. It is evident from a consideration of the data that the three compounds must be combined in a certain ratio for maximum activity. On a molar basis the optimum proportions of cholesterol, oleate and cholic acid are approximately 2:6:1, or 3:6:1 if maximum activity is taken to occur over the range indicated by the horizontal portion of each graph. Somewhat higher activities are attained with the former ratio. Under these conditions maximum activity occurs when 32 μmoles of cholesterol, 96 μmoles of oleate and 16 μmoles of cholic acid are present in a total volume of 2·2 ml. Swell, Field & Treadwell (1953) reported maximum activity when cholesterol, oleic acid and sodium taurocholate were present in a molar ratio of 1:2:1. To obtain the clear emulsion required by their turbidimetric-assay procedure Hernandez & Chaikoff (1957) combined the same three compounds so that the ratio was 1:2:4·5.

Influence of substrate composition and concentration upon rate of hydrolysis. The effect of cholic acid concentration upon rate of hydrolysis of cholesteryl oleate is given in Fig. 4. The optimum

Table 3. *Enzymic synthesis of* [4-^{14}C]*cholesterol ester*

Standard test system for synthesis of cholesteryl oleate was used except that the substrate was prepared to contain about 0·033 μc of [4-^{14}C]cholesterol/ml. The reaction was allowed to proceed for about 2 hr. Part (2 ml.) of the reaction mixture (total volume 2·2 ml.) was extracted in 50 ml. of acetone–ethanol (1:1, v/v). The extract was centrifuged and a portion of the supernatant was used to prepare cholesterol digitonide before and after hydrolysis according to Sperry & Webb (1950). The digitonide was dissolved in acetic acid and was transferred into scintillating fluid for counting. A second portion of the supernatant was dried, and the residue was dissolved in light petroleum and placed on the silicic acid column of Fillerup & Mead (1953) for isolation of free and ester cholesterol. The recovery was about 87%. The eluates were dried and the residue was dissolved in scintillation fluid. All counting was done with a Packard Tri-Carb liquid-scintillation counter. The counting efficiency was standardized by the addition of internal standard to each sample.

	Counts/min./2·2 ml. of reaction mixture			Ester (%)
	Total	Free	Ester	
Isolated as cholesterol digitonide				
Control	40 150	39 600	550*	1·3
Test	40 150	23 320	16 830*	42·0
Isolated as free and esterified cholesterol by chromatography				
Test	34 643*	20 350	14 293	41·5

* Calculated values.

molar ratio of cholesteryl oleate to cholic acid is 5:1. In order to prepare a satisfactory emulsion of this as well as other sterol esters it has been found advantageous to add lithium oleate and albumin, as already indicated. With the complete substrate, maximum activity occurs when 56 μmoles of cholesteryl oleate, 9·6 μmoles of cholic acid, 28 μmoles of lithium oleate and albumin are present in a total volume of 2·2 ml.

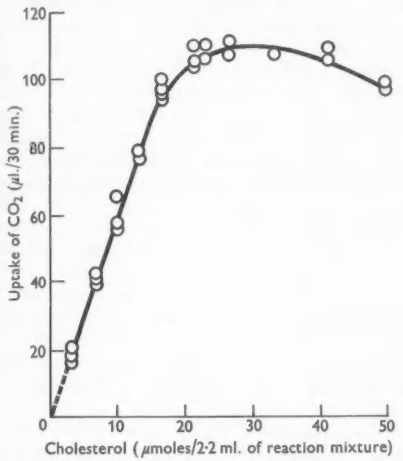

Fig. 1. Effect of cholesterol concentration on rate of cholesteryl oleate synthesis. The standard test system was used except that the substrates were prepared to give the range of cholesterol concentrations shown.

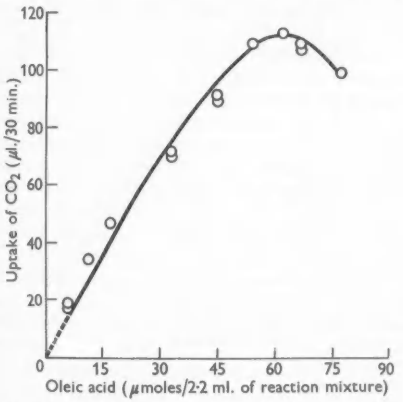

Fig. 2. Effect of oleic acid concentration on rate of cholesteryl oleate synthesis. The standard test system was used except that the substrates were prepared to give the indicated range of oleic acid concentrations.

Effect of various bile acids upon rate of synthesis and hydrolysis. In the preceding sections it has been tacitly assumed that the stimulatory properties of cholic acid are unique. That this is so for both synthesis and hydrolysis of cholesteryl oleate is seen from the data presented in Table 4. None of the common bile acids, either free or conjugated, possesses the effectiveness of cholic acid. All other

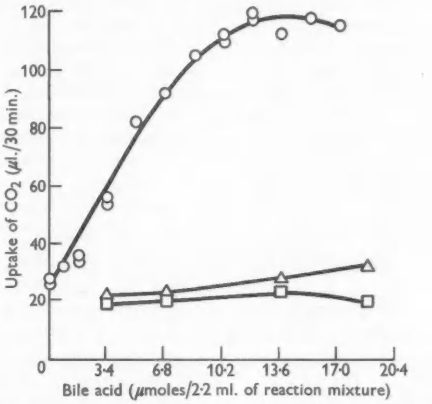

Fig. 3. Rate of esterification as a function of bile acid concentration. The standard test system was used except that substrates were prepared to give the concentrations of cholic acid (○), taurodeoxycholic acid (△) and taurocholic acid (□) shown.

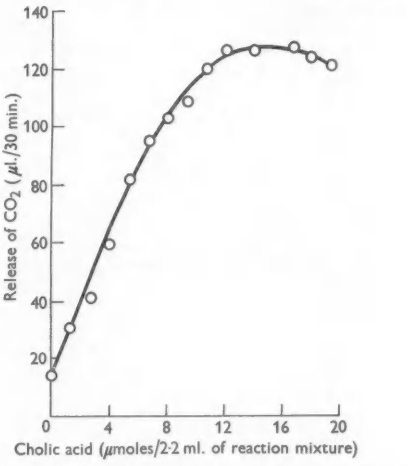

Fig. 4. Effect of cholic acid concentration on rate of cholesteryl oleate hydrolysis. The standard test system was used except that the substrates were prepared to give the required range of cholic acid concentrations.

bile acids tested in the esterifying system are only slightly stimulatory and all to about the same degree. The virtual lack of effect over a considerable range of concentrations is shown for two bile acids in Fig. 3. In the hydrolytic system, conjugates of cholic acid are somewhat more effective than are conjugates of deoxycholic acid. Neither system is stimulated significantly by 3:7:12-trioxocholanic acid. Similar results have been obtained with β-sitosterol.

Effect of ionic environment upon the rate of synthesis and hydrolysis. The activity of an extract of acetone-dried pancreas is considerably increased upon the addition of appropriate amounts of salt to the reaction mixture. Dialysis of enzyme solutions against water invariably results in extensive inactivation. But activity is restored if salt is incorporated into the reaction mixture.

Table 4. *Influence of various bile acids on the rate of synthesis and hydrolysis of cholesteryl oleate*

Standard test systems were used. The bile acids listed were substituted for cholic acid in the standard substrates. The reaction period was 30 min.

Bile acid	Synthesis ($-\mu$l. of CO_2)	Hydrolysis ($+\mu$l. of CO_2)
None	11	4
Cholic	65	124
Deoxycholic	14	13
Lithocholic	14	0
Taurocholic	16	42
Taurodeoxycholic	17	21
Glycocholic	17	49
Glycodeoxycholic	20	20
Glycolithocholic	15	2
3:7:12-Trioxocholanic	17	12

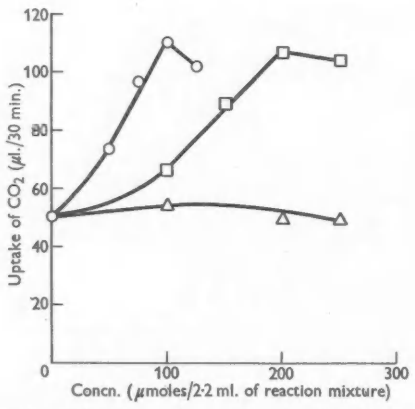

Fig. 5. Effect of ionic environment on rate of esterification. The standard test system was used except that $(NH_4)_2SO_4$ (○), NaCl (□) or dextrose (△) was used in the reaction mixture to give the concentrations shown.

The magnitude of the effect due to salt upon the rate of cholesteryl oleate synthesis is shown in Fig. 5. A suitable ionic environment is established with a variety of electrolytes; the effect is not due to a particular ion. However, un-ionized compounds, as dextrose, are totally ineffective (Fig. 5). According to the data of Fig. 5, 200 μmoles of sodium chloride is equivalent to 100 μmoles of ammonium sulphate. These are optimum concentrations under the conditions imposed; greater amounts cause formation of a precipitate. Effectiveness in this reaction is governed by the total number of cationic and anionic charges. Thus Na^+ ion and NH_4^+ ion are equivalent and 2 Cl^- ions are replaceable by SO_4^{2-} ion. On this basis sodium chloride and ammonium sulphate are equally effective if the former is present at a concentration twice that of the latter. The activating effect of various univalent ions upon synthesis of cholesteryl oleate has also been reported by Hernandez & Chaikoff (1957).

The response of the hydrolytic system to various amounts of sodium chloride or ammonium sulphate indicates that these electrolytes are equally effective if the comparison is made on the basis of ionic strength. Thus 400 μmoles of ammonium sulphate are as effective as 1200 μmoles of sodium chloride. These are optimum concentrations in the standard test system.

Similar results for both systems have been obtained with other electrolytes, although the choice is limited to ions which will not form insoluble compounds with any of the ingredients present in the reaction mixture.

Because it is not possible to prepare a reaction mixture which is devoid of ionizable compounds, considerable activity exists even in the absence of added electrolyte (Fig. 5).

The relationship between substrate concentration and concentration of ammonium sulphate required for maximum activity is not the same for both systems. The amount of ammonium sulphate needed for optimum activity increases as the concentration of substrate for esterification decreases. In the standard test system 0·8 ml. of substrate requires about 100 μmoles of ammonium sulphate. When the volume of substrate is reduced to 0·2 ml., 250 μmoles of ammonium sulphate are needed. At all substrate concentrations tested when the amount of ammonium sulphate exceeds the optimum, a precipitate forms and the activity decreases markedly. In the hydrolytic system the optimum concentration of ammonium sulphate is independent of the substrate concentration (Fig. 6).

The concentration of electrolyte required for maximum esterifying activity is influenced by the nature of the sterol. The amount of either sodium chloride or ammonium sulphate that will permit

maximum activity with β-sitosterol is approximately one-half of that needed with cholesterol.

Effect of pH *upon activity.* The pH optimum for synthesis of cholesteryl oleate is pH 6·1 and for hydrolysis it is pH 7·0. Swell & Treadwell (1950*a*, 1955) reported pH 6·2, as did Hernandez & Chaikoff (1957) for synthesis, and pH 6·6 for hydrolysis of cholesteryl oleate (Yamamoto *et al.* 1949).

Effect of enzyme concentration upon activity. Under the conditions specified, the rate of synthesis and hydrolysis of cholesteryl oleate is proportional to the enzyme concentration within rather narrow limits (Fig. 7).

Relation between sterol structure and rate of esterification with oleate. The use of optimum conditions empirically established for synthesis of cholesteryl oleate to determine the rate of synthesis of other sterol esters may lead to ambiguous conclusions. β-Sitosterol is esterified 37% faster than is cholesterol if the comparison is made with the optimum electrolyte concentration for each, i.e. 23 mM-ammonium sulphate for β-sitosterol and 45 mM-ammonium sulphate for cholesterol, all other conditions being as in the standard test system. If, however, the optimum electrolyte concentration for cholesterol (45 mM-ammonium sulphate) is used, then the rate with β-sitosterol is 43% slower than with cholesterol. This rate, or even a lesser one, has been the usual one reported (Swell, Field & Treadwell, 1954; Hernandez & Chaikoff, 1957). Further, in order to obtain maximum activity with β-sitosterol, the molar ratio of sterol:oleate:cholate must be altered from 2:6:1, as is optimum for cholesterol, to 6:6:1. When this is done, β-sitosterol is esterified about 300% faster than is cholesterol.

The data of Table 5 indicate that under optimum conditions for synthesis of cholesteryl oleate, cholesterol-esterase activity is confined to those sterols in which the steric orientation of the hydroxyl group at C-3 is β, as *epi*cholesterol and *epi*cholestanol are not esterified. Both cholestanol and β-sitostanol are esterified at rates somewhat greater than their respective C-5 unsaturated analogues. Hence, saturation of the double bond at C-5 is permitted but only if rings A and B are joined in the *trans*-decalin manner; no reaction occurs with coprostanol. Hernandez & Chaikoff (1957) have reported similar results. The importance of an intact phenanthrene nucleus and the presence of a hydroxyl group at C-3 is emphasized by the lack of activity with β-norcholesterol and cholesteryl chloride.

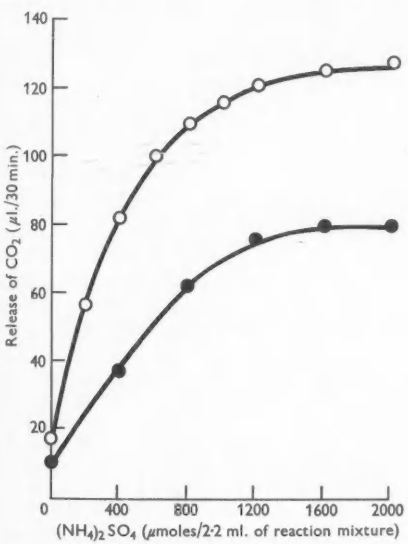

Fig. 6. Effect of substrate concentration on optimum electrolyte concentration in the hydrolytic system. The standard test system was used except that the substrate was used in amounts of 0·9 ml. (○) and 0·3 ml. (●) and the concentration of $(NH_4)_2SO_4$ was varied over the range shown.

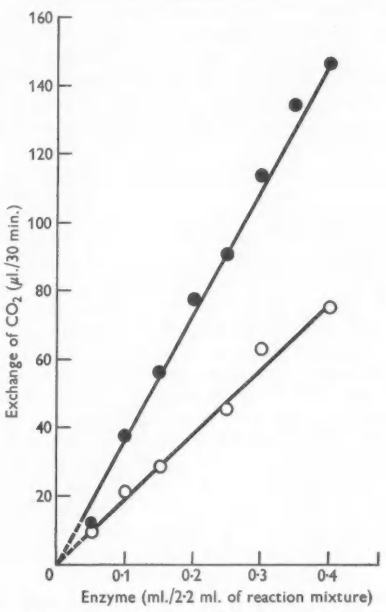

Fig. 7. Rate of synthesis (○) and hydrolysis (●) of cholesteryl oleate as a function of enzyme concentration. The standard test system was used.

Table 5. *Effect of sterol structure on the rate of esterification with oleate*

The standard substrate for esterification was prepared as described but concentration of sterol was reduced to 0·025 M. The standard test system was used except that the amount of substrate per flask was reduced to 0·5 ml.

	$-\mu$l. of CO_2/30 min.
Cholesterol	103
*epi*Cholesterol	5
Cholestanol	139
*epi*Cholestanol	5
Coprostanol	7
β-Sitosterol	71
β-Sitostanol	95
β-Norcholesterol	0
Cholesteryl chloride	0

DISCUSSION

The catalytic activity described in this paper has been attributed to the enzyme known as cholesterol esterase, even though some of the properties obtained by use of the manometric method of estimation differ from those reported by previous workers. The data demonstrate that with the proper ionic environment, pH, bile acid and substrate concentration and composition, the rate of both the synthetic and hydrolytic reactions is so high that it is sufficient to make observations over relatively short periods, during which time linear rates prevail. Furthermore, activity is proportional to enzyme concentration, albeit the range is limited. Conclusions, depending upon differences in rate, have been made on the basis of data obtained under these conditions. Data obtained under conditions requiring long periods of incubation, especially with homogenates, may not be directly comparable.

Although the enzyme is readily obtainable in soluble form and appears susceptible to purification by methods currently in use (Hernandez & Chaikoff, 1957), no firm evidence is yet available which distinguishes cholesterol esterase from other esterases, especially lipase. The definitive answer to this important question must await fractionation. A comparison of some catalytic properties has led Swell *et al.* (1955) to suggest that cholesterol esterase and lipase are unique enzymes.

The beneficial effects of electrolytes, in colloidal systems serving as substrate for enzymic attack, have recently been noted by Mattson & Beck (1955) for lipase and by Hernandez & Chaikoff (1957) for cholesterol esterase. It has been shown in the present report that the concentration of a given electrolyte required for optimum activity is governed by the type of reaction being catalysed, i.e. synthesis or hydrolysis of cholesteryl oleate, and by the nature of the sterol. The latter case deserves particular emphasis inasmuch as it illustrates that differences in sterol structure, however minor, may exert a profound effect upon the physical character of the colloidal substrate of which the sterol is a part. For instance, the electrolyte concentration required to reach maximum rate of synthesis of cholesteryl oleate is about twice that needed for synthesis of β-sitosteryl oleate. Furthermore, the relationship between substrate concentration and the optimum electrolyte concentration differs. In the system for synthesis of cholesteryl oleate, substrate concentration is inversely related to electrolyte concentration, whereas in the hydrolytic system the electrolyte concentration is independent of substrate concentration. These data indicate that the obvious action of the electrolyte is on the substrate, although effects on the enzyme protein are not ruled out. The difference between the responses of the two systems toward electrolyte may indicate dissimilar function, at least in part. It may be that the ionic environment changes the electrical properties of the colloidal substrate in such a manner as to make it more readily accessible to the active centres of the enzyme, as well as permitting the arrangement of the components of the colloidal substrate in a definite way suitable for enzymic attack. In addition, the rate is favourably affected because the optimum concentration of electrolyte for the two systems is so different that the reverse reaction is inhibited, especially at pH values which are not advantageous to either reaction. In the hydrolytic system this occurs because the products of the reaction are removed from colloidal solution, and thus from the sphere of effective enzymic action, whereas the electrolyte concentration in the system for synthesis is too low for rapid hydrolysis. Mattson & Beck (1955) suggest that the stimulation of lipase by high electrolyte concentration is brought about by alteration of the enzyme protein into a more reactive form.

Although the stimulatory effect of bile acids upon the activity of cholesterol esterase has been known since the observations of Mueller (1916*b*), there is no clear evidence in the literature to implicate a particular bile acid as being responsible for the phenomenon. According to the data of Swell *et al.* (1953) both taurocholate and cholate stimulate the synthesis of cholesteryl oleate more effectively than do any of the other common bile acids. Of these two, the former is the more active. In the hydrolytic system taurocholate and cholate are reported as approximately equally effective, although the published data would indicate that cholate is the more stimulatory (Swell *et al.* 1953).

The unique effect of cholic acid, as described, was observed by comparison only with pure bile acids and under conditions such that the bile acid in question was in colloidal solution with the other

components of the substrate. The high specificity of the bile acid requirement in both systems strongly suggests a substrate–enzyme type of relationship. Two properties of bile acids in general are pertinent to consider in the present situation: surface activity and ability to form molecular compounds, even though of low stability. It is tempting to speculate that these two properties not only need to be combined in one chemical substance, but that in addition certain structural features must be present in the molecule to permit interaction with the enzyme protein in proximity to the catalytic site. Interaction with other parts of the enzyme, i.e. non-specific interactions, are not excluded but do not lead to enhanced catalysis. The dual actions of surface activity and molecular-compound formation of the bile acid, occurring near the catalytic site, permit the colloidal substrate to form a complex with the enzyme much more readily than it could in the absence of bile acid or in the presence of those bile acids not fully satisfying the structural requirements for specificity.

SUMMARY

1. The synthesis and hydrolysis of cholesteryl oleate catalysed by pancreatic cholesterol esterase has been followed manometrically in a sodium bicarbonate–carbon dioxide buffer system.

2. The optimum composition, as well as the concentrations of the components, of the colloidal substrate for synthesis and for hydrolysis of cholesteryl oleate has been determined empirically.

3. Data which define the colloidal substrate and other conditions for optimum activity with a particular sterol may not be applicable to a different sterol.

4. The stimulatory capacity of cholic acid, not shared by other bile acids, suggests a relationship with the enzyme of a highly specific nature. Its function is postulated to be that of a specific detergent and former of molecular compounds, acting, by virtue of its unique structure, in the vicinity of the reactive sites.

5. The electrolyte requirement, satisfied by various ions, is governed by the substrate but may also affect the enzyme protein.

6. Cholesterol-esterase activity is confined to sterols with a β-oriented hydroxyl group at C-3, with rings A and B arranged as in *trans*-decalin and with the phenanthrene nucleus intact.

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The Mechanism of Carbohydrase Action

5. ACTION OF HUMAN SALIVARY α-AMYLASE ON AMYLOPECTIN AND GLYCOGEN*

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When human salivary α-amylase acts on whole starch (amylose + amylopectin) there is produced a mixture of fermentable and unfermentable sugars (Myrbäck, 1948). The fermentable sugars consist of maltose, maltotriose and/or glucose (see below). The non-fermentable sugars (α-limit dextrins) are a mixture of higher oligosaccharides. Since the amylose component of starch, which consists essentially only of 1:4-linked α-glucose units, can be converted by the amylase completely into maltose and maltotriose (Whelan & Roberts, 1953) the α-limit dextrins must arise from the amylopectin component and may be assumed to contain the α-1:6-bonds which constitute the points of branching in the dendritic structure of this polysaccharide. The object of this investigation was to characterize the α-limit dextrins and thereby to define the action of salivary α-amylase on amylopectin and the related polysaccharide glycogen. A preliminary account of this work has already appeared (Whelan & Roberts, 1952).

EXPERIMENTAL

Materials

Enzymes. α-Amylase was prepared from human saliva as by Whelan & Roberts (1953), by the method of Meyer, Fischer, Staub & Bernfeld (1948). R-Enzyme was prepared from broad beans ('Prolific Longpod', Sutton and Sons, Reading) as by Hobson, Whelan & Peat (1951).

Substrates. Glycogen was prepared from the livers of pregnant rabbits. The livers were boiled with water as soon as possible after removal from the animal. After cooling, the liver was ground with acid-washed sand and re-extracted with the same liquid by boiling for 20 min. Then the solution was cooled, centrifuged, and the supernatant solution filtered through paper. The glycogen was precipitated with ethanol (2 vol.) and washed with 66% (v/v) ethanol, and ether. Finally it was purified by precipitation with acetic acid (Bell & Young, 1934; Schlamovitz, 1951).

The amylopectin was a gift from Dr T. J. Schoch. This was waxy-maize starch, which is almost pure amylopectin in the sense of being free from amylose and contains little, if any, esterified phosphorus (Schoch, 1953). It was prepared from hand-sorted single-cross Tapicorn seed, (Bear Hybrid Co., Decatur, Illinois, U.S.A.) as by Schoch (1957).

Buffers. These were 0·2M-sodium acetate–acetic acid (pH 7·0) and 0·2M-sodium citrate–citric acid (pH 7·0).

Methods

Digests. Waxy-maize starch was moistened with ethanol and dissolved in 0·6N-sodium hydroxide by heating in a boiling-water bath for 5 min. After cooling, the alkali was neutralized to phenolphthalein either with sulphuric acid [digests (1) and (2), see below] or hydrochloric acid [digest (3)]. Glycogen and the enzymes were dissolved in water. After the substrate had dissolved, the buffer was added and then the enzyme. The digest was covered with toluene or toluene–nitrobenzene (3:1, v/v) and incubated at 35°. After incubation the enzyme was inactivated by heating in a boiling-water bath for 3 min.

Reducing power, developed during the digestions, was measured with the Somogyi (1945*a*) reagent, the time of heating with the reagent being 60 min., in order to develop full reducing power (Whelan, Bailey & Roberts, 1953). When R-enzyme was present the digest portion was treated with Somogyi's (1945*b*) deproteinizing reagents before measuring reducing power. A digest containing buffer and enzyme but no substrate was used in each case to estimate the reducing power of the non-substrate components.

Chromatography. Sugars were examined by chromatography on Whatman no. 54 paper in one or more of the following solvents: water-saturated phenol (Partridge, 1948), pyridine–fusel oil–water (1:1:1, by vol.), propanol–ethyl acetate–water (6:1:3, by vol.) and butanol–acetic acid–water (4:1:5, by vol.). The sugar spots were revealed with aniline hydrogen phthalate (Partridge, 1949), benzidine–trichloroacetic acid (Bacon & Edelman, 1951) or silver nitrate–sodium hydroxide (Trevelyan, Procter & Harrison, 1950).

Charcoal–Celite columns were prepared and used as by Whelan *et al.* (1953). All column dimensions given are those of the adsorbent. Sugars were detected in the fractions by measurement of optical rotation in a 4 dm. tube. All weights of products quoted were estimated by reducing power, where the product was glucose, or by acid hydrolysis to glucose, as by Pirt & Whelan (1951). The weights are quoted in terms of combined glucose ($C_6H_{10}O_5$).

Characterization of products. The products of enzyme action were characterized by one or more of the following methods: measurement of $[\alpha]_D$ (in water) (4 dm. tube), concentration being estimated by acid hydrolysis to glucose; R_F and co-chromatography with authentic material; formation of the β-acetate (Whelan & Roberts, 1953), measuring the $[\alpha]_D$ (in chloroform) and m.p. (un-

* Part 4: Walker & Whelan (1957).

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corrected); degree of β-amylolysis (Whelan & Roberts, 1954); measurement of degree of polymerization (*DP*), based on the reducing power, relative to that of maltose (Whelan *et al.* 1953).

α-*Amylolysis of amylopectin* (1)

Waxy-maize starch (8·063 g., moisture content 13·1%, starch content 6·77 g. by acid hydrolysis) was incubated at 35° in 20 mM-sodium citrate buffer (pH 7·0, 1 l.) with salivary α-amylase (1 g.). After 48 hr. the apparent percentage conversion into maltose was 71·6%. The enzyme was inactivated and a portion of the digest (982 ml., 6·65 g. of starch) was fractionated on a charcoal–Celite column (4·2 cm. × 95 cm.). No monosaccharide was eluted with water (3·3 l.) but di- (2·42 g.), tri- (1·74 g.) and higher oligo-saccharide fractions (1·97 g.) were obtained, a recovery of 92·4%. The di- and tri-saccharide fractions corresponded in R_F values to maltose and maltotriose respectively. The disaccharide had *DP* 2·02, $[\alpha]_D + 134°$, and its β-acetate m.p. 159°, $[\alpha]_D + 63°$. The trisaccharide had *DP* 3·00, $[\alpha]_D + 169°$, and its β-acetate m.p. 136°, $[\alpha]_D + 87°$. These are the properties of maltose, maltotriose and their derivatives (Whelan & Roberts, 1953).

The higher oligosaccharides had *DP* 8·89. A small-scale digestion with α-amylase showed an increase in reducing power. Accordingly, the dextrins (1·88 g.) were incubated at 35° in 8 mM-sodium citrate buffer (pH 7·0, 250 ml.) with salivary α-amylase (0·881 g.). The reducing power of a 0·5 ml. portion of digest rose from 3·90 ml. of 5 mN-thiosulphate (45 min. heating) to 6·00, 6·14, 5·99 and 6·12 ml. after 7, 9, 11 and 14 days respectively. The activity of the enzyme remaining after this time was such that soluble starch was rendered achromic after a few minutes incubation. The enzyme was inactivated and a portion of the digest (234 ml., 1·76 g.) was fractioned on charcoal–Celite (4·0 cm. × 72 cm.). No monosaccharide was eluted with water (3·2 l.). Ethanol (7·5%) eluted maltose (0·103 g.) but 15% ethanol (5·4 l.) did not elute any trisaccharide. Ethanol (50%) desorbed higher oligosaccharides (1·358 g.). The recovery of maltose and oligosaccharides was therefore 82·4%. When corrected for this degree of recovery, assumed to be 82·4% for maltose itself, the total yield of maltose in the two experiments was 2·56 g. The molar ratio maltose:maltotriose (1·74 g., see above) was 2·20:1.

When examined by paper chromatography the higher oligosaccharides showed substances migrating approximately in the positions of malto-pentaose, -hexaose and -heptaose and spots of even lower R_F. The average *DP* was 7·06. When the dextrins (81·5 mg.) were incubated at 35° with R-enzyme (70 mg.) in 12 mM-sodium acetate buffer (pH 7·0, 25 ml.) the reducing power of a 3 ml. portion rose from 5·33 ml. of 5 mM-thiosulphate to 11·47 ml. after 41 hr., remaining constant thereafter, at 2·15 times the initial value.

α-*Amylolysis of amylopectin* (2)

Waxy-maize starch (11·42 g., wet wt., starch content 9·99 g. by acid hydrolysis) was incubated at 35° in 20 mM-sodium citrate buffer (pH 7·0, 1 l.) with salivary α-amylase (1·51 g.) in 1 l. of solution. After 9 days incubation the apparent conversion into maltose was 60·6%. A further amount of enzyme (0·827 g.) was added and the reducing power increased to 71·6% conversion during the next 24 hr. Thereafter an increase of about 0·5% conversion/day took place over 27 days and the reaction was terminated by inactivating the enzyme at 85·6% conversion. The digest (960 ml., 9·56 g. of starch) was fractionated on charcoal–Celite (5 cm. × 118 cm.). Glucose (0·29 g., estimated by optical rotation), maltose (4·64 g.) and maltotriose (1·88 g.) were obtained and their identities checked by measurement of R_F. The α-dextrins (2·60 g.) were eluted with 50% ethanol. When maltotriose was incubated with the salivary α-amylase used in this experiment a slow increase in reducing power took place, amounting to 10% after 59 days.

The α-dextrins had *DP* 7·13 and were not further attacked by α-amylase, when treated under the same conditions as the original amylopectin. They were identical in composition with the first preparation, as revealed by paper chromatography, and the two preparations were combined.

Enzymic debranching of amylopectin α-limit dextrins. The combined α-limit dextrins (see above, 3·47 g.) were incubated at 35° in 20 mM-sodium acetate buffer (pH 7·0, 500 ml.) with R-enzyme (0·924 g.). After 140 and 168 hr. the apparent conversion into maltose was constant at 62·0%. The initial value was 28·3% and the ratio final: initial reducing power was therefore 2·19:1. A portion of the digest (8 ml.) was then incubated for 24 hr. with R-enzyme (50 mg.), when no further increase in reducing power took place. Accordingly, the enzyme in the main digest was inactivated and a portion (465 ml., 3·22 g.) fractionated on charcoal–Celite. No glucose was found. Maltose and maltotriose were obtained in pure form but the last fractions containing the triose also contained maltotetraose and similarly some of the tetraose was contaminated with pentaose. The pentaose was followed by higher oligosaccharides, mainly hexaose and heptaose, which were combined together. To estimate the yields of individual components of mixed fractions the mixtures were treated with β-amylase under conditions which would hydrolyse maltotetraose and higher maltodextrins but not maltotriose (Whelan *et al.* 1953). The products were quantitatively analysed on charcoal–Celite (Whelan & Roberts, 1953; Whelan & Roberts, 1954). In the case of the maltotetraose–maltopentaose mixture, for example, the latter yields maltotriose on β-amylolysis, the former does not, but both produce maltose. Accordingly, from the amounts of maltose and maltotriose produced on β-amylolysis the composition of the original mixture could be assessed. The total yields of the saccharides produced from the α-limit dextrins and their properties are reported in Table 1. These properties, together with the correspondence of the R_F values of these substances with authentic maltodextrins, serve to justify their description as maltodextrins.

α-*Amylolysis of amylopectin* (3)

Waxy-maize starch (13·03 g., moisture content 7·15%, starch content 11·13 g. by acid hydrolysis) was incubated at 35° in 20 mM-sodium citrate (pH 7·0, 1 l.) with salivary α-amylase (1 g.). After dissolving the starch in alkali the solution had been neutralized with hydrochloric acid, not, as in Expts. (1) and (2), with sulphuric acid (see Methods). After 19 days the apparent conversion into maltose was 73·6%. More enzyme (0·510 g.) was added, and after 33, 43 and 48 days the conversion was constant at 76·8%. After inactivating the enzymes the digest (985 ml.; 10·99 g. of starch) was fractionated on charcoal–Celite (4·0 cm. ×

120 cm.) as usual. Glucose (81 mg.), maltose (4·73 g.) and maltotriose (3·00 g.) were obtained. The column was then eluted with 23% ethanol (4·5 l.), 25% ethanol (3 l.), 27·5% ethanol (4 l.), 40% ethanol (4 l.) and 50% ethanol (4 l.), fractions of 100 ml. or 200 ml. being collected. The fractions reserved for examination were *A* (eluted between 1·3 l. and 1·6 l. by 23% ethanol), *B* (3·0–3·8 l., 23% ethanol) and *C* (13·4–14·4 l. 40% ethanol).

Fraction A. This was a single substance migrating slightly more slowly on paper than maltopentaose. It had *DP* 4·85. A portion (10 mg.) was treated with R-enzyme (1 ml., 10 mg.) for 48 hr. at 35°. When fractionated on paper, substances migrating with maltose and maltotriose were seen.

Fraction B. As judged by R_F, this contained mainly heptasaccharide(s) with a small amount of hexasaccharide(s). It had *DP* 6·82. A portion (20 ml., 100 mg.) was reduced with sodium borohydride (1·5 ml., 22 mg.) for 3 hr. at room temperature. Unchanged borohydride was decomposed with acetic acid and the solution was neutralized and diluted to 25 ml. Of this, 20 ml. were incubated with R-enzyme (65 mg., 1 ml.) and acetate buffer (pH 7·0, 3 ml.). The reducing power became constant between 46 and 69 hr. Assuming the dextrin to be singly branched the reducing power at this time was 85·5% of that expected if complete debranching had occurred. The digest was examined by paper chromatography in comparison with that of an unreduced sample, also treated with R-enzyme. The observations are described in the Results section.

Fraction C. This had *DP* 14·1 and when treated with R-enzyme as above the reducing power of portions of the digest after 16·5 and 40 hr. were 8·98 and 9·15 ml. of 5 mN-thiosulphate, compared with an initial value of 2·63 ml. The mean final reducing power of 9·07 ml. therefore represented an increase of 3·44 times.

α-Amylolysis of glycogen

Rabbit-liver glycogen (7·75 g., glycogen content 7·26 g. by acid hydrolysis) was incubated at 35° in 20 mM-sodium citrate (pH 7·0, 1 l.) with salivary α-amylase (0·950 g.) for 9 days. The apparent conversion into maltose was then 42·0%. After adding more enzyme (0·750 g.) the conversion increased by about 2% per day until a value of 56·4% was reached, remaining constant for a further 8 days. After inactivating the enzyme the digest (7·53 g. of glycogen) was fractionated on charcoal–Celite (4·0 cm. × 120 cm.). No glucose was obtained. Maltose (and maltulose) (1·82 g.) were eluted with 7·5% ethanol and the higher dextrins (4·50 g.) were desorbed with 55% ethanol. These dextrins (4·39 g.) were incubated at 35° in 1 l. of 20 mM-acetate buffer (pH 7·0) with R-enzyme (2·88 g.) for 39 hr. The reducing power rose from 40·7 to 58·4%, as maltose. The addition of more enzyme (1·44 g.) did not change the reducing power appreciably, the values after a further 48 and 72 hr. being 59·1 and 59·2% respectively. The enzyme was inactivated and a portion of the digest, equivalent to 4·25 g. of the original glycogen, was fractionated on charcoal–Celite (4·0 cm. × 80 cm.) as in Expt. 2 for waxy-maize starch (see above). No glucose was obtained and the disaccharide fraction contained only maltose. The yields and properties of the products are in Table 2.

A second α-amylolysis of glycogen was performed to determine the amounts of maltose and maltotriose obtainable by α-amylolysis. (In the first experiment the maltotriose was not separated from the α-limit dextrins before they were debranched.) From 6·16 g. of α-amylolysed glycogen at an apparent conversion into maltose of 60·3% there were obtained 2·05 g. of maltose (+maltulose) and 1·51 g. of maltotriose, a molar ratio of 2·01 : 1.

Table 1. *Properties of the maltodextrins obtained on debranching the amylopectin α-limit dextrins*

DP represents degree of polymerization, based on reducing power relative to that of maltose. Figures in parenthesis are theoretical degrees of amylolysis (Whelan, Bailey & Roberts, 1953; Whelan & Roberts, 1953, 1954).

Dextrin	Yield (mg.)	*DP*	$[\alpha]_D$ in water	Degree of amylolysis (%) α-Amylase	Degree of amylolysis (%) β-Amylase
Maltose	526	2·00	135°	—	—
Maltotriose	785	2·94	159	—	98 (100)
Maltotetraose	1028	3·84	173	95 (100)	91 (100)
Maltopentaose	211	4·92	179	81 (80)	78 (80)
Higher dextrins	295	6·68	183	—	85

Total yield 2·845 g., from 3·22 g. of α-dextrins.

Table 2. *Properties of the maltodextrins obtained on debranching the glycogen α-limit dextrins*

Dextrin	Yield (mg.)	*DP*	$[\alpha]_D$ in water	Degree of amylolysis (%)* α-Amylase	Degree of amylolysis (%)* β-Amylase
Maltose	561	2·00	135°	—	—
Maltotriose†	1930	3·00	154	—	94
Maltotetraose	852	4·00	164	92	85
Maltopentaose	215	4·92	174	81	74
Higher dextrins	247	6·11	—	—	83

Total 3805 g., from 4·25 g. of α-dextrins.

* See Table 1.

† This figure includes the maltotriose liberated in the initial α-amylolysis of the glycogen and not separated from the α-limit dextrins before debranching (see Experimental section).

Calculation of the degree of branching of amylopectin and glycogen

Method 1. When the α-limit dextrins of amylopectin were debranched with R-enzyme the reducing power rose to 215% of the original value (see Expt. 1). Therefore the dextrins (*DP* 7·06) contained 1·15 branch links per molecule. The yield of dextrins from 6·65 g. of amylopectin was 1·99 g., allowance being made for incomplete recoveries of material (92·4%, 82·4%) in the two fractionations required for the isolation of the dextrins. The only other products of the α-amylolysis were maltose and maltotriose. All the branch links of the amylopectin were therefore 'concentrated' in the α-limit dextrins. The number of glucose units for each enzymically hydrolysable branch point in the original amylopectin is therefore given by $(6{\cdot}65/1{\cdot}99) \times (7{\cdot}06/1{\cdot}15) = 20{\cdot}4$.

In a branched molecule of high molecular weight such as amylopectin the number of branch points should equal the number of non-reducing-end groups. The number of such end groups determined by periodate oxidation of the same sample of amylopectin was 1 per 24·4 glucose residues (Peat, Whelan & Thomas, 1956).

Method 2. The number of molecules of α-limit dextrins can be calculated from the yields of maltodextrins obtained after debranching (Tables 1 and 2). If all the α-limit dextrins contained only 1 branch point per molecule this molar quantity would be half that of the maltodextrins. Limit dextrins with higher degrees of branching were, however, also present. To make an approximate calculation of the amount of these dextrins two assumptions were made, first that there were no α-limit dextrins containing more than two branch points, and secondly that the maltodextrins greater in size than maltopentaose ('higher dextrins', Tables 1 and 2) represented the base chains of the doubly branched dextrins. (The base chain is the sequence of 1:4-linked α-glucose units carrying the reducing group.) If the molar quantity of maltose–maltopentaose is M_1 and of higher maltodextrins is M_2, then the molar quantity of α-limit dextrins is $M_2+(M_1-2M_2)/2 = M_1/2$, and the 'molar' quantity of branch points is

$$2M_2+(M_1-2M_2)/2 = M_2+M_1/2.$$

The values in Table 1 correspond to 2·88 'm-moles' of branch points. These are associated with a total quantity of glucose residues in the maltodextrins of $2845/162 = 17{\cdot}5$ m-moles (Table 1). The number of glucose residues per branch point is therefore $17{\cdot}5/2{\cdot}88 = 6{\cdot}08$. This is to be compared with the value of $7{\cdot}06/1{\cdot}15 = 6{\cdot}13$, determined by method 1. By further calculations made as in method 1 the number of glucose residues per branch point in the original amylopectin is 20·3. In view of the agreement of this value with that derived by method 1 the error introduced by the assumptions made in this second method of calculation does not seem to be of any consequence.

The same calculations can be made for glycogen. The results in Table 2 have to be adjusted to allow for the fact that the maltotriose produced by α-amylase was not separated from the α-limit dextrins before they were debranched with R-enzyme. The amount of maltotriose formed by α-amylase was determined in a separate experiment, described above.

The number of glucose residues per branch point of the α-limit dextrins, calculated from the quantities of maltodextrins formed from the limit dextrins by R-enzyme, is 6·07, the same as for amylopectin. The calculated proportion of branch links in the whole glycogen molecule is 1 per 12·0 glucose residues. By periodate oxidation [semi-micro method of Peat, Whelan & Turvey (1956)] Dr J. R. Turvey found the non-reducing end-group content of this glycogen to be 1 per 12·8 glucose residues.

RESULTS AND DISCUSSION

α-Amylolysis of waxy-maize starch

Amylopectin was treated with α-amylase and when the rate of reaction had become very low the products were examined by paper chromatography in propanol–ethyl acetate–water. Substances were seen which migrated with maltose and maltotriose but a spot corresponding to maltotetraose was absent. After maltotriose, in order of descending R_F, was a substance migrating slightly more slowly than maltopentaose, followed by a series of spots extending to the origin. Large-scale separation of the digest components enabled the maltose and maltotriose to be characterized and identified while the larger molecules were collected as a single fraction, having an average *DP* 8·89. These were found still to be susceptible to α-amylolysis, the reducing power being raised by about 70% on further treatment with enzyme. When this digestion mixture was fractionated maltose was obtained, but not maltotriose. The higher oligosaccharides were no longer susceptible to α-amylolysis, and had *DP* 7·06. Glucose was not detected in the first or second digestions, either on paper chromatography of the whole digest or during the large-scale fractionation. The molar ratio total maltose:maltotriose was 2·20:1. This is different from the ratio (2·39:1) of the two sugars produced from amylose (Whelan & Roberts, 1953). Taken with the fact that maltotriose was not formed at all in the second digestion the results indicate that the 1:6-branch points of amylopectin, not present in amylose, exert a steric effect on the course of the enzyme action on those parts of the molecule giving rise to these two sugars.

A second α-amylolysis of amylopectin was performed which was prolonged much beyond the time of the first experiment. In this case a small amount of glucose was obtained, with maltose, maltotriose and α-limit dextrins. It seemed that the glucose was formed from the maltotriose and not from the α-limit dextrins. There were three reasons for this conclusion. First, the α-limit dextrins had the same *DP* (7·13) as those in the first experiment, when glucose was not formed. Secondly, the paper-chromatographic pattern of the two limit-dextrin preparations was the same. Thirdly, assuming the glucose to have arisen from maltotriose, the molar ratio maltose:maltotriose

from this hydrolysis would have been 2·21:1, the same as in the first experiment.

When the two limit-dextrin preparations were combined and treated with R-enzyme to hydrolyse the 1:6-linkages, the products proved to be maltose, malto-triose, -tetraose, -pentaose and higher maltodextrins (Table 1).

Action pattern of salivary α-amylase on amylopectin

It is evident that the α-1:6-branch links of amylopectin confer resistance to α-amylolysis on α-1:4-bonds in their vicinity. If this were not so then isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) would be found as a product of the amylolysis. The fact that the smallest α-limit dextrin found was a pentasaccharide suggests that three α-1:4-bonds close to the branch link are rendered stable to α-amylase action. This pentasaccharide was split by R-enzyme into maltose and maltotriose. There are five ways in which these two sugars can be joined to each other through an α-1:6-link and in formulating a hypothesis of α-amylase action we assumed previously that the pentasaccharide had the most symmetrical of these structures, that in which maltose is joined to the central glucose unit of maltotriose, i.e. 6^2-α-maltosylmaltotriose [Whelan & Roberts (1952); nomenclature as by Whelan (1960)]. Structural analysis of the pentasaccharide, however, proved the maltose to be joined to the non-reducing-end unit of maltotriose, i.e. the pentasaccharide is 6^3-maltosylmaltotriose (Fig. 2*a*, Bines & Whelan, 1960). The amylase-resistant links are therefore not symmetrically disposed around the branch link but are situated as in Fig. 1; the hypothesis of α-amylase action therefore needs to be revised.

Under the conditions employed in these experiments salivary α-amylase rapidly hydrolyses non-terminal α-1:4-bonds of starch but does not attack the terminal bonds at the reducing or non-reducing chain ends (Whelan & Roberts, 1953). Maltotriose, containing only terminal bonds, is therefore resistant to α-amylolysis and only begins to break down under conditions of prolonged enzyme action (digestion no. 2) or with relatively high concentrations of enzyme (Walker & Whelan, 1960). An α-limit dextrin formed under the chosen experimental conditions will therefore be one in which the α-1:4-bonds are resistant to amylolysis either by reason of their proximity to the 1:6-link or because they occupy terminal positions. There are eight structures which fit this requirement (Fig. 2). These are the pentasaccharide (Fig. 2*a*), of proven structure, and a range of hexa- to octa-saccharides, whose structures have been derived from that of the pentasaccharide by adding one or more terminal-linked glucose units. Each of these complies with the above definition of an α-limit dextrin. In these dextrins the base chain, i.e. that carrying the reducing group, varies in length from 3 to 5 glucose units, and the side chain from 2 to 3 glucose units. On debranching with R-enzyme the α-limit dextrins should therefore give rise to maltose, malto-triose, -tetraose and -pentaose, and these were in fact found. In addition, however, maltohexaose and higher maltodextrins were also found but their presence can be accounted for (see below). Further evidence in support of the hypothesis was obtained by subfractionating the α-limit dextrins to yield a heptasaccharide component. When this was debranched with R-enzyme the products were maltose, malto-triose, -tetraose and -pentaose. (The triose and tetraose were the major components of the mixture.) This agrees with the predicted products from dextrins *e*, *f* and *g* (Fig. 2). When the heptasaccharide was reduced with sodium borohydride and then debranched the only reducing sugars formed were maltose and maltotriose. The side chains of the dextrin were therefore maltose and maltotriose and the base chains maltotetraose and -pentaose. This again agrees with prediction.

It remains to account for the formation of maltohexaose and larger maltodextrins on debranching the α-limit dextrins. The reason for this was suggested from the fact that during the debranching process the reducing power was more than doubled, increases of 2·15 and 2·19 times being noted in two experiments. This was suggestive of the presence of dextrins containing more than one branch point. Such dextrins were obtained by subfractionating the α-limit dextrins. The particular fraction examined had average *DP* 14·1 and on debranching the reducing power increased 3·44 times. Evidently there were dextrins with two, three and perhaps more branch points per molecule. These dextrins would be expected to arise from regions of the molecule in which the branch points are so close that all intervening links are resistant to α-amylase. In constructing possible formulae for such dextrins it became evident that to satisfy the

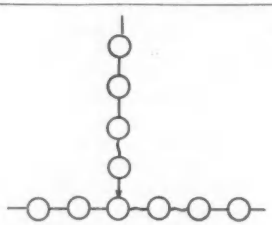

Fig. 1. A segment of the amylopectin or glycogen molecule in the vicinity of a branch point, showing the positions of α-1:4-links resistant to α-amylase. ○, α-Glucose unit; —, hydrolysable 1:4-link; ∼, resistant 1:4-link; ↓, 1:6-link.

requirement that they should contain maltohexaose and higher maltodextrins within the molecule they would have to exhibit multiple branching. By this is meant the occurrence of two or more C_6-substituted glucose residues in a maltodextrin chain and waxy-maize starch is known to contain multiple branches (Peat, Whelan & Thomas, 1952, 1956). Fig. 3 shows the largest α-limit dextrins containing two and three branch links per molecule which the hypothesis permits. Both the singly- and multiply-branched molecules are shown. The singly-branched dextrins do not contain a maltodextrin chain longer than five units but the upper limit is nine for the multiply-

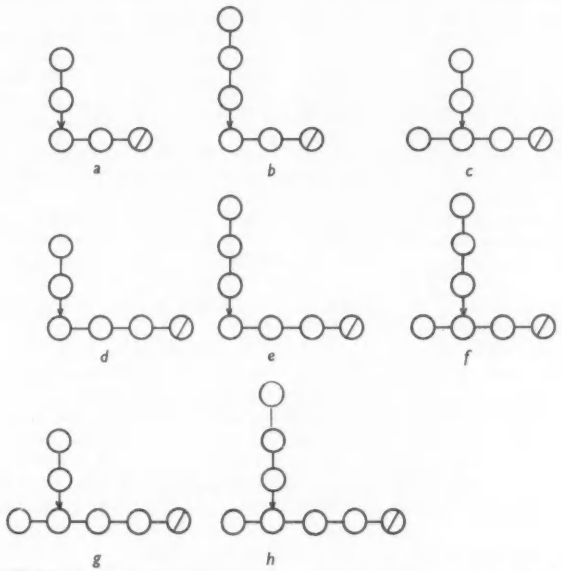

Fig. 2. Singly-branched α-limit dextrins of amylopectin and glycogen. ○, α-Glucose unit; ⊘, reducing-end glucose unit; —, 1:4-link; ↓, 1:6-link.

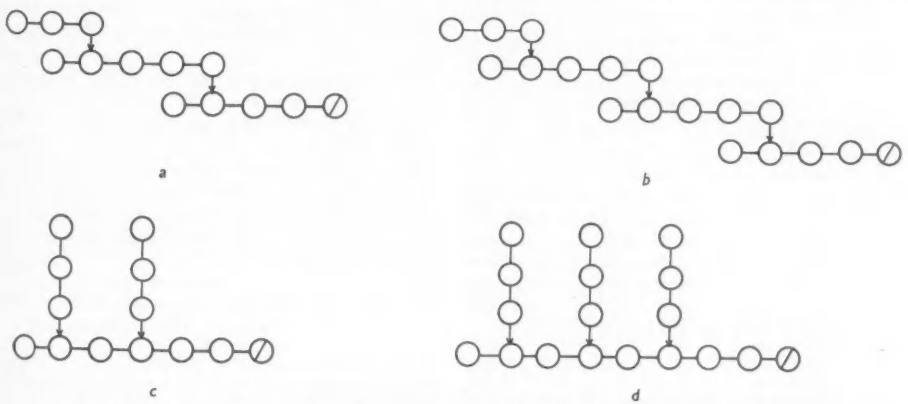

Fig. 3. Structures of the largest doubly- and triply-branched α-limit dextrins exhibiting single (*a*, *b*) and multiple (*c*, *d*) branching. Symbols as in Fig. 2.

branched dextrins. The presence of the maltodextrins of $DP > 5$ in the debranched α-limit dextrins is therefore consistent with our hypothesis of salivary α-amylase action.

α-Amylolysis of glycogen

The pattern of the α-amylolysis of glycogen seems to be the same as for amylopectin. Maltose, maltotriose (molar ratio 2·03:1) and α-limit dextrins of the same type as from amylopectin are formed and the products of debranching of the dextrins are the same (Table 2). Glucose was not found after α-amylolysis or after debranching with R-enzyme, but a disaccharide product was found which was not encountered, except in very small amount, with amylopectin. This was maltulose (4-*O*-α-D-glucopyranosyl-D-fructose) and this finding has already been reported (Peat, Whelan & Roberts, 1952). The yield of maltulose varied in different experiments according to the source of the rabbit-liver glycogen and its origin is the subject of continuing investigation.

Correlation of this work with other studies of α-amylolysis

The products we have characterized as resulting from the α-amylolysis of amylopectin and glycogen, i.e. maltose, maltotriose and α-limit dextrins are end products of α-amylolysis in the sense that when tested with a concentration of enzyme similar to that used in their preparation no further breakdown took place. By using far greater amounts of enzyme, with the incorporation of chloride ions to activate the enzyme, the maltotriose and α-limit dextrins can be caused to undergo further hydrolysis (Walker & Whelan, 1960). The products described here, except maltose, are not therefore true end products, but the attainment of the levels of α-amylolysis at which these substances persist in a metastable condition is readily reproducible, as evidenced by the number of such experiments reported here. The difficulty of defining the true limit of α-amylolysis of amylopectin and glycogen is discussed by Walker & Whelan (1960). The level of amylolysis achieved here seems to represent the lowest in what is possibly a series of discrete stages of amylolysis, readily reproducible and defined in terms of the conditions of the experiment, i.e. concentration of enzyme and time of incubation. A recognition of the existence of these stages in the enzyme action serves to explain why different workers report different 'end products' of α-amylolysis (Walker & Whelan, 1960).

Proportions of 1:6*-links in amylopectin and glycogen*

The isolation and analysis of the products of α-amylolysis and of enzymic debranching of the α-limit dextrins were performed as far as possible on a quantitative basis. It thus became possible to calculate the proportions of 1:6-branch links in amylopectin and glycogen. Two methods were used and are described in the Experimental section. The values so obtained were in reasonable agreement with the estimates of non-reducing end-group content made by periodate oxidation. This measure of agreement permits three conclusions. First, R-enzyme splits all the branch points in the α-limit dextrins. This is in contrast with its incomplete action on amylopectin and its almost complete lack of action on intact glycogen (Peat, Whelan & Thomas, 1956; Peat, Whelan, Hobson & Thomas, 1954). Secondly, α-amylase does not split any of the branch points susceptible to R-enzyme action. If it did then the determined proportion of branch points would be lower than of non-reducing end groups. Thirdly, if, as is believed (Hobson *et al.* 1951), R-enzyme is specific for the hydrolysis of α-1:6-links, then most, if not all, of the branch links of amylopectin and glycogen are of this type.

SUMMARY

1. Amylopectin (waxy-maize starch) is degraded by low concentrations of human salivary α-amylase to yield maltose (42%), maltotriose (28%) and α-limit dextrins containing the α-1:6-branch links of the amylopectin. Glycogen gives the same products.

2. The smallest α-limit dextrin is a pentasaccharide, the α-1:6-link of which is split by R-enzyme to yield maltose and maltotriose. α-Limit dextrins containing two and three branch links per molecule are also present.

3. The maltodextrins formed by R-enzyme action on the mixed α-limit dextrins have been separated, identified and analysed. A hypothesis to explain the formation of the limit dextrins has been formulated. It is suggested that under the chosen experimental conditions three α-1:4-bonds in defined positions adjacent to the branch link are resistant to α-amylase action. The hypothesis accounts for the presence of the α-limit dextrins containing more than one branch link only if the parent polysaccharides exhibit multiple branching.

4. By quantitatively analysing the products of α-amylolysis and those formed on subsequently debranching the α-limit dextrins, the proportions of α-1:6-links in amylopectin and glycogen have been calculated. The results agree with the measured proportions of non-reducing-end groups and it is concluded that salivary α-amylase does not split the α-1:6-links in these polysaccharides.

We thank Professor Stanley Peat, F.R.S., for his encouragement and advice, Dr T. J. Schoch for a gift of waxy-maize starch and the Department of Scientific and Industrial Research for a maintenance grant (to P.J.P.R., 1950–53).

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Biochem. J. (1960) **76**, 253

The Mechanism of Carbohydrase Action

6. STRUCTURE OF A SALIVARY α-AMYLASE LIMIT DEXTRIN FROM AMYLOPECTIN

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Roberts & Whelan (1960) studied the action of a low concentration of human salivary α-amylase on amylopectin and at the limit of the reaction found the products to be maltose, maltotriose and a series of larger molecules, termed α-limit dextrins. These were split by R-enzyme, believed to be specific for the hydrolysis of α-1:6-glucosidic linkages (Hobson, Whelan & Peat, 1951), into maltodextrins. The α-limit dextrins therefore contained the amylase-resistant branch linkages of the original polysaccharide. The smallest of these dextrins is a pentasaccharide (Peat, Whelan & Roberts, 1956) and this communication describes its structural analysis. A preliminary report has already been made (Whelan & Bines, 1955).

EXPERIMENTAL

Methods. All evaporations were carried out under reduced pressure at about 40°. Measurements of optical rotation were made in a 4 dm. tube. R_F values of sugars were measured in butanol–acetic acid–water (4:1:5, by vol.) or propanol–ethyl acetate–water (6:1:3, by vol.), and of methylated sugars in butanol–ethanol–water–ammonia (sp.gr. 0·88) (40:10:19:1, by vol.). Sugar spots were revealed with benzidine–trichloroacetic acid (Bacon & Edelman, 1951) or silver nitrate–sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Ionophoresis was carried out in 0·2 M-boric acid–0·05 M-borax (2:3, v/v; pH 8·7) and M_G values calculated as by Foster (1953). Charcoal–Celite columns were prepared as by Whelan, Bailey & Roberts (1953). Solutions of the pentasaccharide used for measurement of optical rotation and of pentasaccharide, panose and isomaltotriose (Turvey & Whelan, 1957) for periodate oxidation were treated with Somogyi's (1945) deproteinizing reagents before use. This was because these sugars had been isolated from charcoal–Celite columns (see Whelan *et al.* 1953). The concentrations of these solutions were determined by acid hydrolysis to glucose (Pirt & Whelan, 1951).

Preparation of methyl sugars. 2:3-Di-*O*-methyl-D-glucose was prepared as by McCloskey & Coleman (1945) and 2:3:6-tri-*O*-methyl-D-glucose as by Haworth, Hirst & Webb (1928). Other methyl sugars used were kindly given by Drs D. J. Bell, E. J. Bourne and A. G. Ross.

Preparation of the pentasaccharide. The pentasaccharide was isolated from digestion mixtures of waxy-maize starch

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and purified human salivary α-amylase prepared by Dr P. J. P. Roberts and described by Roberts & Whelan (1960). Fractions from several such digests were combined and refractionated on charcoal–Celite to yield about 1 g. of chromatographically and ionophoretically pure material having $[\alpha]_D^{20}+181°$ in water (*c*, 0·08). Maltopentaose has $[\alpha]_D+180°$ (Whelan *et al.* 1953). The dextrin was resistant to salivary α-amylase when tested as for maltodextrins as by Whelan & Roberts (1953).

Methylation experiments

Methylation of the pentasaccharide. The pentasaccharide (720 mg.) was first methylated as by Albon, Bell, Blanchard, Gross & Rundell (1953), being dissolved in 50% (v/v) aqueous dioxan and treated successively with dimethyl sulphate (3·65 ml.) and 30% (w/v) aqueous sodium hydroxide (7·3 ml.) at room temperature. The mixture was stirred for 15 hr., when all reducing power had disappeared. Sodium hydroxide (8 ml.) and dimethyl sulphate (4 ml.) were then added alternately at 10 min. intervals until totals of 80 and 40 ml. respectively had been added, when, after a further 20 min., the solution was heated on a boiling-water bath for 30 min. to decompose methyl sulphates. After cooling, the solution was neutralized with sulphuric acid. Most of the sodium sulphate was precipitated by adding ethanol (1 vol.) and storing at 0° overnight. The filtrate was evaporated to dryness and the residue again treated with the methylating agents. In all, three such methylations were performed and after the third methylation the neutralized reaction mixture was extracted with chloroform (3 × 50 ml.) to yield 855 mg. of soluble methylated sugar. This was methylated 10 times as by Purdie & Irvine (1903). In each treatment the dextrin was refluxed with methyl iodide (1·54 g., 10·8 m-moles) and silver oxide (1·20 g., 4·5 m-moles) for 4 hr. The iodide was distilled away and the residue extracted with chloroform, filtered, and the filtrate evaporated, the syrupy residue then being remethylated. Finally the syrup was dissolved in ethanol, filtered and evaporated, then in water, and filtered and evaporated. The residue weighed 520 mg. (56%) (Found: OMe, 49·3%. The fully methylated pentasaccharide, $C_{47}H_{86}O_{26}$, requires OMe, 49·4%).

Hydrolysis and fractionation of the methylated pentasaccharide. The methylated pentasaccharide (500 mg.) was heated in 0·33 N-sulphuric acid (500 ml.) for 10 hr. at 100° and then, because tests showed hydrolysis to be incomplete, for a further 12 hr. in N-sulphuric acid. After this time the reducing power had attained a constant value. The solution was neutralized with sodium hydroxide and evaporated to 100 ml. The solution (α_D 1·462°) was adsorbed on a charcoal–Celite column and subjected to gradient elution. A reservoir of water (4 l.) was connected to the top of the column and kept at constant level by the automatic addition of 30% aqueous ethanol, with stirring. Fractions (100 ml.) were collected under gravity. Fig. 1 shows the progress of the elution. After removal of fraction *D* the eluting solvent was changed to 25% aqueous ethyl methyl ketone, when material having a total rotation of +0·088° in 100 ml. was collected. This was not further examined. The total α_D of all fractions was +1·301°, a recovery, in terms of rotation, of 91·3%. The fractions corresponding to each peak (*A*, *B*, *C* and *D*) were combined and evaporated to dryness.

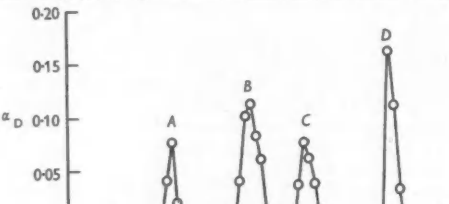

Fig. 1. Fractionation of the hydrolysate of the methylated pentasaccharide on charcoal–Celite. Details are given in the Experimental section. *A*, 2:6-Di-*O*-methyl-D-glucose; *B*, 2:3:6-tri-*O*-methyl-D-glucose; *C*, 2:3:4-tri-*O*-methyl-D-glucose; *D*, 2:3:4:6-tetra-*O*-methyl-D-glucose.

Identification of fraction A. This fraction behaved as a homogeneous substance during paper-chromatographic and -electrophoretic analysis and had R_P 0·51 and M_G 0·00. The R_P of 2:3-di-*O*-methylglucose was 0·57 and the M_G of the 3:6-isomer was 0·82. The sugar was thought likely to be the 2:6-isomer, in which case treatment with phenylhydrazine would yield 6-*O*-methylglucosazone. The sugar (30 mg.) was heated at 100° with phenylhydrazine (70 mg.), sodium bisulphite (10 mg.) and 0·2 M-sodium acetate–acetic acid buffer (pH 4·8, 5·0 ml.) for 24 hr. (Bell & Manners, 1954). The solution was evaporated and applied to 40 cm. lines drawn on six filter papers (Whatman no. 54, 18¼ in. × 22½ in.). The papers were subjected to electrophoresis at 400 v for 6 hr. in 0·2 M-sodium acetate–acetic acid buffer (pH 4·8) when the phenylhydrazine salts moved away from the origin, leaving a yellow band of osazone, which was cut out and extracted with ethanol. This solution was concentrated and applied to six filter papers (as above) which were irrigated with butanol–acetic acid–water (4:1:5, by vol.). The osazone moved with the solvent front, and was completely separated from the salts. The coloured bands were eluted from the air-dried papers with ethanol and the extract evaporated to a crystalline residue (11·4 mg., 21%) having m.p. 182–183°, $[\alpha]_D^{20}$ −74·0→ −45·0° (90 hr., const.) in ethanol (Found: N, 15·0. Calc. for $C_{19}H_{24}O_4N_4$: N, 15·0%). 6-*O*-Methylglucosazone prepared as by Freudenberg & Hüll (1941) had m.p. 185–186°, $[\alpha]_D^{20}$ −74·2°→ −45·8° in ethanol. A mixture of the two specimens melted at 185°.

Identification of fraction B. This fraction crystallized on being evaporated to dryness and was further crystallized twice from *iso*propyl ether–chloroform (9:1, v/v). It had m.p. 121–122°, $[\alpha]_D^{20}+68·7°$ in ethanol (*c*, 0·16). Authentic 2:3:6-tri-*O*-methylglucose had m.p. 121°, $[\alpha]_D^{20}+69·5°$, the m.p. being unchanged when mixed with fraction *B*.

Identification of fraction C. A portion of the syrup (68 mg.) was kept at 30° for 45 hr. with aniline–ethanol (1·1 ml.; 2:3, v/v). Water (10 ml.) was added and distilled away. The syrup crystallized on standing and was recrystallized four times from light petroleum (b.p. 40–60°)–ether (1:1, v/v). The crystals had m.p. 145–145·5°, $[\alpha]_D^{19}-102°$ in ethanol (*c*, 0·045) (Found: C, 61·0; H, 7·93; N, 4·74. Calc. for $C_{15}H_{23}O_5N$: C, 60·6 H, 7·74; N, 4·72%). Authentic 2:3:4-tri-*O*-methylglucose anilide had m.p. 146°, $[\alpha]_D-103°$ and was unchanged in m.p. on being mixed with the anilide of fraction *C*.

Identification of fraction D. The syrupy residue crystallized on standing and was twice recrystallized from light petroleum (b.p. 40–60°)–ether (9:1, v/v). A portion (47·6 mg.) was converted into the anilide by heating at 100° with aniline (0·30 ml.) and ethanol (0·45 ml.) for 3 hr. Crystals were formed on cooling and were twice recrystallized from ethanol. The compound had m.p. 136–137°, $[\alpha]_D^{20}$ +233° in ethanol (*c*, 0·07) (Found: N, 4·72. Calc. for $C_{18}H_{23}O_5N$: N, 4·50%). 2:3:4:6-Tetra-*O*-methylglucose anilide had m.p. 136–137°, $[\alpha]_D^{20}$ +235° and was unchanged in m.p. when mixed with the anilide of fraction *D*.

Acid-catalysed demethylation of methylglucoses. 0·1% Solutions of 2:3-di-*O*-methylglucose (325 mg.), 2:3:6-tri-*O*-methylglucose (262 mg.) and 2:3:4:6-tetra-*O*-methylglucose (413 mg.) in 0·33 N-sulphuric acid were heated at 100° for 10 hr., and then in N-acid for a further 12 hr., as in the hydrolysis of the methylated pentasaccharide (see above). The solutions were neutralized and then adsorbed on and eluted from charcoal–Celite as for the pentasaccharide. In terms of optical rotation the recoveries of di-, tri- and tetra-*O*-methylglucoses were 91·3, 95·5 and 93·7% respectively. All fractions collected before the emergence of the main sugar peak were combined, evaporated and dissolved in 100 ml. of water for determination of optical rotation. These rotations correspond to 2·1, 2·4 and 1·2% respectively of the rotations of the di-, tri- and tetra-*O*-methylglucoses before treatment with acid. Paper-chromatographic examination of these fractions showed the probable presence of mono-*O*-methylglucose in the product from di-*O*-methylglucose, mono- and di-, and mono-, di- and tri-*O*-methylglucoses in the other two products respectively.

Periodate-oxidation experiments

Periodate oxidations and measurements of formaldehyde were carried out as by O'Dea & Gibbons (1953) and O'Dea (1953) except that the ratio sugar solution:*p*-hydroxybenzaldehyde:periodate–bicarbonate was 9:1:10 (by vol.). AnalaR D-glucose was used as the standard source of formaldehyde. The results are shown in Table 1.

RESULTS AND DISCUSSION

Acid and enzymic hydrolysis of the pentasaccharide

When a partial acid hydrolysate of the pentasaccharide was fractionated by paper chromatography two disaccharides were found, having the R_F values of maltose and isomaltose. The only monosaccharide present was glucose. Roberts & Whelan (1960) had previously found that R-enzyme, believed to be specific for the hydrolysis of α-1:6-glucosidic linkages (Hobson *et al.* 1951), split the pentasaccharide into two sugars, having the R_F values of maltose and maltotriose. The tentative conclusion that the pentasaccharide was one of the five structures pictured in Fig. 2 enabled experiments to be designed to decide between the various alternatives.

Methylation analysis of the pentasaccharide

The pentasaccharides in Fig. 2 can be divided into two groups. Methylation and hydrolysis of one group (*Q*, *R*, *T*) should yield 2:3:4:6-tetra-, 2:3:6-tri- and 2:3-di-*O*-methylglucose in the molar ratio 2:2:1. The second group (*P*, *S*) should yield the first two of these sugars with 2:3:4-tri-*O*-methylglucose, in the molar ratio 1:3:1. The pentasaccharide was exhaustively methylated, apparently to the extent calculated for complete etherification. On hydrolysis and fractionation (Fig. 1) four methyl-sugars were isolated and identified. These were 2:3:4:6-tetra-, 2:3:6-tri-, 2:3:4-tri- and 2:6-di-*O*-methylglucose. The unexpected feature of the experiment was the isolation of the dimethylglucose, since its formation could be interpreted as meaning the presence in the pentasaccharide of 1:3-links. Wolfrom & Thompson (1956) have indeed isolated nigerose (3-*O*-α-glucosylglucose) from partly hydrolysed waxy-maize amylopectin and Hamilton & Smith (1956) have found that the same amylopectin contains 0·2–0·5% of glucose units resistant to periodate oxidation. These could be 1:3-linked units. An alternative explanation of the origin of the dimethyl-sugar was the de-etherification of the tri- and tetra-methylglucose during acid hydrolysis of the methylated pentasaccharide. Control experiments showed, however, that the amount of de-etherification occurring could not account for the 14% (molar basis) yield of the 2:6-isomer. A third possibility was incomplete methylation of the pentasaccharide and we are inclined to this as the correct explanation. (In this case the apparently full degree of methylation of the pentasaccharide, as shown by the methoxyl content, could have been due to adhering traces of ethanol.) It is known that the C-3 hydroxyl group of glucose is much less easily substituted than are the other hydroxyl groups. Full methylation of phenyl α-glucoside should give the 2:3:4:6-tetra-*O*-methyl derivative, but the reaction at the C-2, C-4 and C-6 hydroxyl groups proceeds so much more

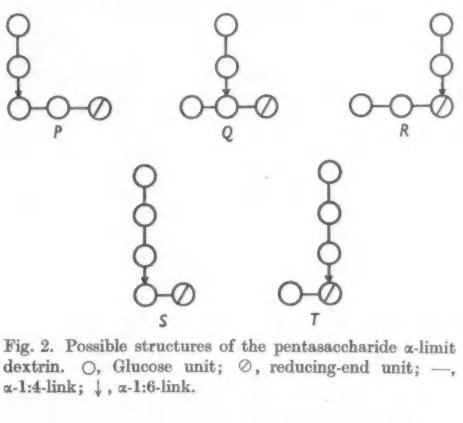

Fig. 2. Possible structures of the pentasaccharide α-limit dextrin. ○, Glucose unit; ⊘, reducing-end unit; —, α-1:4-link; ↓, α-1:6-link.

rapidly than at C-3 that the direct methylation of this glucoside is a practical method for preparing 2:4:6-tri-*O*-methylglucose (Richtmyer, 1939). 2:6-Di-*O*-methylglucose was obtained from methylated glycogen, and as a result 1:3-links were claimed to be present (Bell, 1948); this was later withdrawn (Bell & Manners, 1954), as also was the claim for 1:3-links in inulin based on the isolation of 2:4:6-tri-*O*-methylglucose from methylated inulin (Hirst, McGilvray & Percival, 1950; Aspinall & Telfer, 1953). The 2:6-di-*O*-methylglucose appeared to arise as a result of incomplete methylation of those glucose residues which should, on full methylation, have given the 2:3:6-tri-*O*-methyl-sugar. There was no sign in the hydrolysate of 2:4:6-tri- or 2:4-di-*O*-methylglucose, which have R_F values distinctly different from any of the four products *A*–*D*. These two sugars would have been formed if the glucose units giving 2:3:4:6-tetra- and 2:3:4-tri-*O*-methylglucose respectively had been incompletely methylated. On this assumption the experimental molar ratio 2:3:4:6-tetra-:2:3:6-tri-+2:6-di-:2:3:4-tri-*O*-methylglucose of 1:2·8:1·1 indicates that the pentasaccharide molecule contains one non-reducing-end group and one 1:6-link and that it must have structure *P* or *S* (Fig. 2).

Periodate oxidation of the pentasaccharide

A method of distinguishing between structures *P* and *S* was devised, based on the over-oxidation reaction of periodate. The same method was developed independently at the same time by Hough & Perry (1956) and since these authors have explained the basis of the method this will not be elaborated here. Structure *P* would be expected to be eroded by periodate from the reducing end of the molecule, the reaction progressing towards the non-reducing end until arrested at the 1:6-linkage. Two glucose units, the reducing-end unit and that next to it, would be completely converted into formic acid, formaldehyde and carbon dioxide before the reaction ceased, the yield of formaldehyde amounting to 2 mol.prop. Structure *S*, on the other hand, would yield only 1 mol.prop. of formaldehyde. Model substances, maltose, panose, melibiose and isomaltotriose, were tested under oxidation conditions described by O'Dea & Gibbons (1953) and O'Dea (1953), namely in periodate–bicarbonate at 50°. The calculated yields of formadehyde were 2, 1, 0 and 0 mol.prop. respectively. The experimental results showed, however, that at 50° the reactions with panose, melibiose and isomaltotriose progressed beyond their expected limits (Table 1). When the temperature was lowered to 20° the abnormal reaction did not take place (Table 1) and the pentasaccharide was accordingly oxidized under these conditions. The yield of formaldehyde was 1·86 mol.prop. and from maltose was 1·80 mol.prop. (calc. 2 mol.prop.). It was clear that structure *P* and not *S* represented the constitution of the pentasaccharide.

The splitting of the pentasaccharide by R-enzyme into maltose and maltotriose shows the structure to be restricted to one of the five substances *P*–*T* (Fig. 2). Only one of these, substance *P*, would yield more than 1 mol.prop. of formaldehyde. The conclusion that the pentasaccharide is substance *P* can therefore be reached independently of the methylation analysis.

The same conclusion can be formed independently of the R-enzyme experiment, provided that the explanation of the origin of the 2:6-di-*O*-methylglucose in the methylation experiment is correct. The results of methylation analysis show that the

Table 1. *Yields of formaldehyde on periodate oxidation of oligosaccharides*

The methods are given in the Experimental section.

Time (min.)	Formaldehyde (mol.prop.) formed at 50°			
	Maltose	Panose	Melibiose	Isomaltotriose
10	1·82	0·74	1·02 (100 min.)	0·88 (100 min.)
30	1·98	1·14	—	—
50	2·00	1·70	—	—
70	2·02	1·70	—	—
90	2·00	—	—	—

Time (hr.)	Formaldehyde (mol.prop.) formed at 20°			
	Maltose	Panose	Melibiose	Pentasaccharide
4	—	0·91	—	—
6	—	1·08	—	—
12	1·47	1·06	—	—
16	1·80	1·06	—	—
18	1·80	1·06	0·09	1·85
41	—	—	—	1·87
45	—	—	—	1·86

molecule contains one non-reducing end group, one 1:6-linked glucose unit and three 1:4-linked glucose units. The high positive $[\alpha]_D$ of the pentasaccharide shows all the links to have the α-configuration. There are four molecules which can be constructed from this evidence. These are structures *P* and *S* (Fig. 2), 6^4-α-glucosylmaltotetraose and 6-α-maltotetraosylglucose [nomenclature as defined by Whelan (1960)]. The respective yields of formaldehyde from these sugars would be 2, 1, 3 and 0 mol.prop. Structure *P* is therefore singled out as the only one consistent with all the facts, and the pentasaccharide is therefore *O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose (6^3-α-maltosylmaltotriose).

SUMMARY

1. A pentasaccharide α-limit dextrin formed from amylopectin by human salivary α-amylase has been subjected to structural analysis.

2. Methylation analysis shows the pentasaccharide to contain one non-reducing-end group and one 1:6-link.

3. The results of periodate oxidation indicate that the 1:6-linkage is the antepenultimate bond, relative to the reducing end of the molecule.

4. The foregoing evidence, taken with the results of acid and enzymic hydrolysis and the high positive rotation of the pentasaccharide, shows the molecule to be 6^3-α-maltosylmaltotriose.

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The Mechanism of Carbohydrase Action

7. STAGES IN THE SALIVARY α-AMYLOLYSIS OF AMYLOSE, AMYLOPECTIN AND GLYCOGEN

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Conflicting views have been expressed on the nature of the end products of the action of human salivary α-amylase on amylose (see review by Whelan, 1958). Whelan & Roberts (1953) recovered maltose and maltotriose in high yield (92·4%) as the only detectable products of the action of a purified enzyme preparation on amylose. There is general agreement that maltose is entirely resistant to the enzyme, but it has been disputed whether maltotriose is a stable end product; it could be further hydrolysed to maltose and glucose. Hopkins & Bird (1954), using unpurified saliva,

stated that 'no glucose was ever detected in actions of salivary amylase on amylose' but that maltotriose was slowly hydrolysed by large amounts of the enzyme. Myrbäck and coworkers (Myrbäck & Leissner, 1943; Svanborg & Myrbäck, 1953) also concluded that salivary amylase does not attack maltotriose. On the other hand, Pazur (1953) found the sugar was attacked by crude saliva. Jansen & Wydeveld (1958) reported the hydrolysis of *p*-nitrophenyl α-maltoside by saliva and concluded that maltotriose would also be hydrolysed. We have examined two possible explanations of these results: either an enzyme distinct from the starch-splitting amylase hydrolyses the maltotriose, or the failure to detect such hydrolysis results from using too low a concentration of enzyme. It is clear that if the amylase does hydrolyse maltotriose there must be an enormous disparity in the rate of this action as compared with that on the next higher maltodextrin homologue, maltotetraose. Whelan & Roberts (1953) reported the complete hydrolysis of maltotetraose under conditions in which there was no detectable breakdown of maltotriose. It is already known, however, that phosphorylase (Whelan & Bailey, 1954) and β-amylase (Whelan, Bailey & Roberts, 1953) each attack these two sugars at greatly different rates, so that hydrolysis of maltotriose by pure α-amylase might occur when sufficient enzyme is used. With the same specimen of crystalline enzyme used in the present work Pazur & Budovich (1955) reported such hydrolysis. The present work confirms and amplifies this finding and deals with a similar question about products of the α-amylolysis of amylopectin and glycogen, from which, in addition to glucose, maltose and maltotriose, salivary α-amylase liberates molecules containing the α-1:6-branch linkages of the polysaccharide (α-limit dextrins) (Roberts & Whelan, 1960). The reported formation of different α-limit dextrins can be attributed to the use of different amounts of enzyme.

MATERIALS AND METHODS

Enzymes. Except in one instance (Table 3) saliva was collected from the same subject (W. J. W.), diluted with an equal volume of water, covered with toluene and centrifuged after 1 hr. to remove mucins. The diluted saliva was dialysed in cellophan against running tap water and then running distilled water at room temperature, each dialysis lasting at least 24 hr. Human salivary α-amylase, crystallized four times, was a gift from Dr Jytte Muus (Muus, 1953). All digests were incubated at 30° under a layer of toluene.

Substrates. Maltose hydrate was supplied by Thomas Kerfoot Ltd., Vale of Bardsley, Lancs. Maltotriose and maltotetraose were prepared as by Peat, Whelan & Kroll (1956). The starch used in determining α-amylase activity was prepared from AnalaR soluble starch as by Hanes & Cattle (1938). The unmodified starch was used in other experiments requiring whole starch. The amylopectin was a gift from Dr T. J. Schoch. Rabbit-liver glycogen was prepared as by Schlamovitz (1951). Oyster glycogen was a commercial sample (British Drug Houses Ltd.). Some properties of these three polysaccharides are given by Walker & Whelan (1960), who used the same specimens. Amylose dextrin was prepared from potato amylose (Hobson, Pirt, Whelan & Peat, 1951). The amylose (2 g.) was dissolved in alkali (Peat, Pirt & Whelan, 1952), neutralized and diluted to 500 ml. Dialysed saliva (5 ml.) was added and the digest incubated at 30° for 11 min. The enzyme was inactivated by heating for 3 min. in a boiling-water bath. Precipitated amylose was filtered away and amylose in solution precipitated with acetone (2 vol.), recovered on the centrifuge, dissolved in the minimum of water and dialysed as for saliva.

Except for maltose, which was assumed to be the pure hydrate, all substrate concentrations were determined by hydrolysis to glucose (Pirt & Whelan, 1951).

Buffers. Phosphate–citrate buffers (McIlvaine, 1921) were used in the determination of optimum pH of enzyme activity; otherwise the buffers were made from citric acid–sodium hydroxide, sodium acetate–acetic acid, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–hydrochloric acid or triethanolamine–hydrochloric acid.

Reducing-power determinations. The copper reagent of Shaffer & Hartmann (1921) was used.

Amylase activity. The method of Schwimmer (1947) and Schwimmer & Balls (1949) was used, except that the pH of the digest was 6·3, not 4·7, and sodium chloride was used in place of calcium chloride.

Paper chromatography. The methods were as given by Walker & Whelan (1960). Before being applied to the chromatograms the digests were heated at 100° for 3 min. to inactivate the enzyme and then freed from inorganic matter with Bio-Deminrolit mixed-bed resin (The Permutit Co. Ltd.) in the carbonate form (Woolf, 1953).

RESULTS

Action of crude and crystalline salivary α-amylase on maltotriose and maltose

Dialysed saliva was allowed to act on maltotriose in the presence of chloride ion, which activates salivary α-amylase. The concentration of enzyme was about 100 times that used by Whelan & Roberts (1953) in the hydrolysis of amylose. A substantial hydrolysis of maltotriose was observed during 22 hr., the rate of reaction being the same in three different buffers (Table 1).

Dialysed saliva was then heated in the presence and absence of chloride to destroy some of the amylase. Digestions of maltotriose carried out in chloride with the same amount of amylase, measured by its action on starch, each resulted in about the same degree of hydrolysis of maltotriose (Table 2). Next, dialysed saliva from two subjects was incubated with maltotriose, with and without chloride ion, equipotent amounts of enzyme in

Table 1. *Action of dialysed saliva on maltotriose*

The digests contained 0·4% of maltotriose, dialysed saliva (0·27 unit/ml.), and 40 mM-buffer, pH 7·0. Sodium chloride, to 20 mM, was added to the citrate digest; the other buffers already contained chloride.

Buffer	Hydrolysis of maltotriose (%) 6 hr.	22 hr.
Citrate	10	33
Tris	12	31
Triethanolamine	12	30

Table 2. *Action of heat-inactivated saliva on maltotriose*

Dialysed saliva was heated at 56·5° for 15, 35 and 60 min., when the activities towards starch were 55, 34 and 18% respectively of the original value. Another sample of saliva, containing 10 mM-sodium chloride, was heated at 63·5° for 25 and 73 min., when the activities were 73 and 47% respectively of the original. Digests were prepared from these samples containing 0·08% of maltotriose, 40 mM-citrate (pH 6·5), 80 mM-sodium chloride and α-amylase (0·6 unit/ml.). Reducing powers were measured initially and after 4·7 hr. Figures in parentheses indicate the time of heating (min.) of the enzyme.

Hydrolysis of maltotriose (%) Enzyme heated without chloride	Enzyme heated with chloride
31 (0)	27 (0)
32 (15)	32 (25)
35 (35)	31 (73)
36 (60)	

Table 3. *Comparison of the actions of saliva and crystalline α-amylase on maltotriose*

(*a*) The digests contained 0·4% of maltotriose, dialysed saliva (0·075 unit/ml. with chloride present, 0·225 unit/ml. with chloride absent), and 50 mM-citrate buffer, pH 6·5. Sodium chloride, when present, was 0·1 M. Dialysed saliva (G.J.W.) initially contained 1·5 units/ml. and dialysed saliva (W.J.W.) 2·7 units/ml. Incubation was for 48 hr.

Saliva	Hydrolysis of maltotriose (%) Chloride present	Chloride absent
G.J.W.	40	10·1
W.J.W.	37	9·7

(*b*) The digests contained 0·28% of maltotriose, dialysed saliva or crystalline α-amylase (0·068 unit/ml.), buffer and chloride as in (*a*), and were incubated for 24 hr.

	Hydrolysis of maltotriose (%)
Dialysed saliva	21
Crystalline enzyme	22

terms of starch hydrolysis being used. Hydrolysis of maltotriose took place in all cases, but much more in the presence of chloride (Table 3*a*). Finally, equipotent amounts of dialysed saliva and crystalline α-amylase hydrolysed maltotriose in the presence of chloride to the same degree (Table 3*b*).

When dialysed saliva acted on maltose under the conditions described in Table 2, maltose being used at the same molarity as that specified for maltotriose, the degree of hydrolysis in 24 hr. was 16%, in both the presence and absence of chloride, each result being the mean of four experiments. Crystalline salivary α-amylase did not act on maltose under these conditions.

In these reactions there was no evidence of transglycosylation. Crude or crystalline α-amylase was incubated with maltotriose or maltose as in Table 2, with added [^{14}C]glucose, and the digests were fractionated by paper chromatography. No radioactivity was detected in the oligosaccharides.

Effect of chloride ions on the α-amylolysis of maltotriose, maltotetraose and starch

The action of dialysed saliva on maltotriose, maltotetraose and starch in chloride at concentrations varying from μM to M was investigated. The chloride concentration in the preparation of dialysed enzyme was not measured but must have been very low since starch and maltotetraose hydrolysis was activated by added chloride in the range 1–10 μM (Fig. 1). The degree of amylolysis of these two substrates was affected in a similar manner by chloride, but no activation of the hydrolysis of maltotriose was noticed until the chloride concentration was raised to 10 mM.

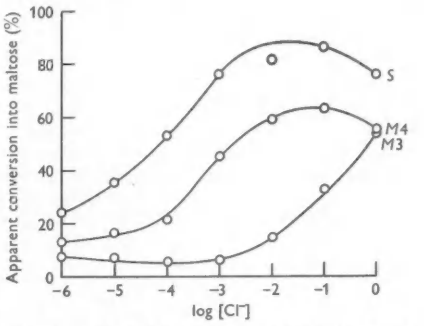

Fig. 1. Effect of chloride on the α-amylolysis of maltotriose (*M* 3), maltotetraose (*M* 4) and starch (*S*). The digests contained 0·4% of substrate, 40 mM-citrate buffer (pH 6·5), dialysed saliva (0·26 unit/ml.) and sodium chloride in concentrations from μM to M. The starch and maltotetraose digests were incubated for 5 min. and maltotriose for 26·5 hr.

Amylolysis of starch and maltotetraose, but not the hydrolysis of maltotriose, was inhibited by 0·1–1·0 M-chloride (Fig. 1). The small (5–8 %) hydrolysis of maltotriose taking place over the chloride-concentration range μM–mM was confirmed by paper chromatography of the digests, which showed the formation of maltose and glucose from the maltotriose.

Optimum pH *of α-amylase action*

Starch and maltotetraose were incubated with dialysed saliva in the presence and absence of chloride at various hydrogen-ion concentrations. With chloride, the pH optimum for starch was 6·5–7·0 and for maltotetraose 6·5. Without chloride the optimum was at a rather more acid pH and was less clearly marked (Fig. 2). Experiments with maltotriose were performed with dialysed saliva and crystalline amylase, no great difference between the two preparations being noted. The results for the crystalline enzyme are given in Fig. 3. With chloride the optimum pH was at pH 5·5. In contrast with starch and maltotetraose hydrolysis the pH optimum without chloride was at a more alkaline pH.

Stages in the α-amylolysis of amylose, amylopectin and glycogen

Amylose was subjected to a slight degree of α-amylolysis and the amylose dextrin isolated (see above). This was preferred to native amylose as a substrate for quantitative studies because of the retrogradation which takes place when undegraded amylose is treated with α-amylase in the absence of chloride (Whelan & Roberts, 1953). The amylose dextrin was treated, in parallel with maltotriose, with crystalline α-amylase in two concentrations, in the presence and absence of chloride. The ratio of the two concentrations of enzyme was 25:1. At the lower concentration, with chloride, the hydrolysate of amylose dextrin attained a constant reducing power, equivalent to an apparent conversion of 95 % of the amylose dextrin into maltose (Fig. 4). In the corresponding maltotriose digest there was a negligible hydrolysis after 72 hr. The 95 % conversion is close to that of 90·8 % noted by Whelan & Roberts (1953) after the α-amylolysis of amylose, which yielded only maltose and maltotriose as end products. At the higher amylase concentration, with chloride the curve of the reaction with amylose dextrin was inflected at about 95 % conversion, then rising slowly to a constant value of 115 % after 45 hr. In the corresponding maltotriose digest the sugar was completely hydrolysed after 72 hr. Paper chromatograms were made of the four 72 hr. digests containing chloride. At the high enzyme concentration the amylose dextrin and maltotriose digests contained maltose and glucose with a very faint trace of maltotriose. At the low concentration the amylose dextrin digest contained maltose and maltotriose with a trace of

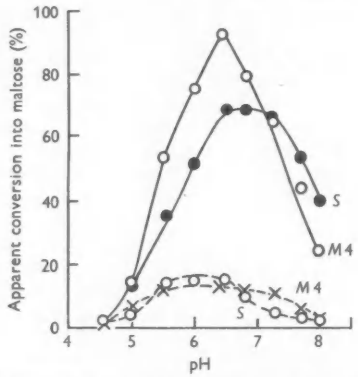

Fig. 2. Effect of hydrogen-ion concentration on the α-amylolysis of starch (*S*) and maltotetraose (*M* 4) in the presence (—) and absence (- - -) of chloride ion. The starch digests contained 1·32 % of starch, 40 mM-phosphate–citrate buffer, dialysed saliva (0·01 unit/ml.) and 0·1 M-sodium chloride, when present. Incubation was for 1 hr. The maltotetraose digest contained 0·44 % of maltotetraose, 50 mM-buffer, dialysed saliva (0·17 unit/ml.) and 0·1 M-sodium chloride, when present. Incubation was for 5 min.

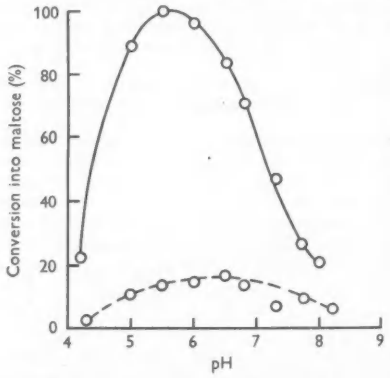

Fig. 3. Effect of hydrogen-ion concentration on the α-amylolysis of maltotriose. When chloride was absent (- - -) the digests contained 0·4 % of maltotriose, 50 mM-phosphate–citrate buffer, crystalline α-amylase (0·41 unit/ml.), and with 0·1 M-chloride present (—) the digests contained 0·24 % of maltotriose, 23 mM-phosphate–citrate buffer and 0·13 unit of crystalline enzyme/ml. Incubation was for 43·5 hr. The activity of the enzyme was tested after 24 hr. and in each case was within 2 % of the amount added.

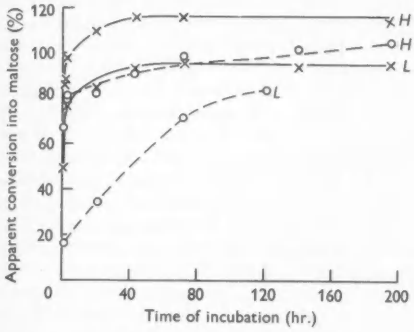

Fig. 4. α-Amylolysis of amylose dextrin with high (*H*) and low (*L*) concentrations of enzyme in the presence (—) and absence (- - -) of chloride. The digests contained 0·36% of dextrin, 29 mM-citrate buffer (pH 6·5), dialysed saliva (0·25 or 0·01 unit/ml.) and 0·1 M-sodium chloride, when present. Maltotriose was treated similarly and the results are reported in the text.

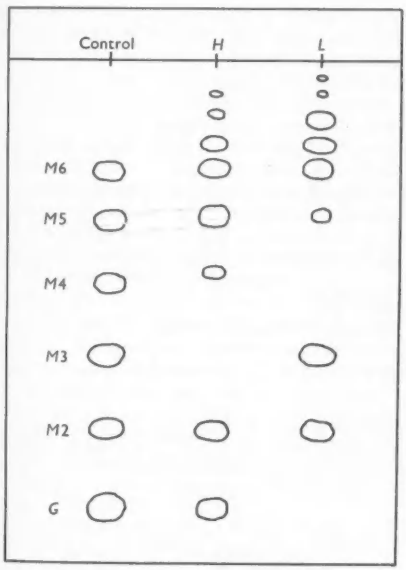

Fig. 5. Paper-chromatographic fractionation of the products of α-amylolysis of glycogen. Rabbit-liver glycogen was treated for 48 hr. with high (*H*) and low (*L*) concentrations of crystalline α-amylase in the presence of chloride as in Fig. 4. The percentage conversions into maltose were 88 and 60% respectively. The reference substances on the chromatogram are glucose (*G*), maltose (*M* 2), maltotriose (*M* 3) etc.

glucose, and the maltotriose digest contained the trisaccharide and barely detectable amounts of maltose and glucose. After 193 hr. incubation of the four amylose dextrin digests the enzyme was still active in each case.

The curves for digests without chloride had similar trends to those with chloride, but the rates of reaction were much lower (Fig. 4). After 72 hr. only 12% of maltotriose was converted at the higher enzyme concentration and none at the lower.

Two samples of glycogen and one of amylopectin were subjected to α-amylolysis by high and low concentrations of enzyme in the presence of chloride, as in Fig. 4. After 48 hr. incubation the digests were applied to paper. The results of the chromatography of amylolysed rabbit-liver glycogen are shown in Fig. 5, and were the same with oyster glycogen and waxy-maize starch as substrates. At high enzyme concentration the products, identified by R_F, were glucose, maltose and a series of sugars, the fastest moving of which had approximately the R_F of maltotetraose. At the low enzyme concentration the products were maltose, maltotriose and a sugar series with a substance having approximately the R_F of maltopentaose as its fastest-moving member.

DISCUSSION

Action of α-amylase on maltotriose and maltose

High concentrations of crude and crystalline salivary α-amylase hydrolysed maltotriose (Tables 1–3), confirming the results of Pazur (1953) and Pazur & Budovich (1955). The rate of hydrolysis relative to that of starch was, however, so low (see below) that four experiments were performed to find whether the maltotriose-splitting activity was due to an enzymic impurity. The results were as follows: (i) Like the hydrolysis of starch, that of maltotriose is activated by chloride ion (Table 3*a*). (ii) Two subjects had the same amount of maltotriose-splitting activity in their saliva, relative to starch-splitting activity (Table 3*a*). This is unlikely if the two activities were those of different enzymes. (iii) No significant change in maltotriose-splitting activity relative to starch-splitting activity occurred when the amylase was partly inactivated by heat (Table 2). (iv) Crude saliva and four-times-crystallized α-amylase had the same ratio of hydrolytic activities with both starch and maltotriose (Table 3*b*). It is reasonable to conclude that maltotriose is hydrolysed by salivary α-amylase.

It has been suggested that since there is only one starch-splitting enzyme in saliva (Meyer, Duckert & Fischer, 1950) it is justifiable to ascribe products arising from the action of saliva on starch to the action of α-amylase (Pazur, 1953). This, however, is

not valid as far as the production of glucose is concerned because saliva contains a second enzyme, forming glucose from maltose. The action seems to be one of hydrolysis rather than of transglycosylation, since no transfer to [^{14}C]glucose was observed, and formation of glucose was accompanied by an increase in reducing power. Chloride did not activate the reaction and crystalline amylase did not hydrolyse maltose. The hydrolysis was therefore due to an enzyme other than α-amylase.

The actions of salivary α-amylase on starch and maltotetraose are very similar, both in respect of optimum pH and the effect of chloride on the rate of reaction (Figs. 1, 2). With maltotriose, however, the pH optimum in the presence of chloride is distinctly different from that for starch and maltotetraose hydrolysis (Figs. 2, 3), and the relative rates of hydrolysis of these two substrates as compared with maltotriose vary in different ways as the chloride concentration is changed (Fig. 1). Although it seems proven that the amylase does hydrolyse maltotriose, the rate of its action on this molecule is of an entirely different order from that on larger molecules. Starch and maltotetraose, representing the two extremes of molecular size on which the enzyme acts rapidly, are not hydrolysed at greatly different rates (Fig. 1). However, whereas 63% of maltotetraose was hydrolysed in 5 min. in 0·1 M-sodium chloride, only 33% of maltotriose was hydrolysed by the same amount of enzyme in 26·5 hr. (Fig. 1). There is a close parallel here with phosphorylase and β-amylase. These enzymes also act on maltotetraose and starch at rather similar rates, but act on maltotriose at very much lower rates (Whelan, 1958).

Stages in the α-amylolysis of amylose, amylopectin and glycogen

The vast difference in the rate of α-amylolysis of maltotriose, as compared with larger substrate molecules, makes it clear that the α-amylolysis of amylose proceeds in two stages. The first stage is the conversion of the amylose into maltose and maltotriose. The second stage involves the hydrolysis of the maltotriose into maltose and glucose. By the use of the appropriate amount of enzyme the reaction can be stopped, or nearly so, at the first stage (Fig. 4). This was the reaction described by Whelan & Roberts (1953) and Hopkins & Bird (1954). The second stage, where all the amylose has become maltose and glucose, is apparently the final arrest point in the reaction.

A recognition of these two clearly separated stages in the α-amylolysis of amylose helps in an understanding of the α-amylolysis of the branched polysaccharides amylopectin and glycogen. As with amylose, there are reports of different end products. Roberts & Whelan (1960) found that the products of the α-amylolysis of amylopectin and glycogen are maltose, maltotriose and a series of α-limit dextrins. These dextrins consist of maltodextrins joined to each other through the α-1:6-branch links of the parent polysaccharide, the smallest being a pentasaccharide. This is 6^3-α-maltosylmaltotriose (Fig. 6*b*) [Bines & Whelan (1960), nomenclature as defined by Whelan (1960)]. Nordin & French (1958), however, reported the products to be glucose, maltose and α-limit dextrins, of which the smallest is a tetrasaccharide (6^3-α-glucosylmaltotriose, Fig. 6*a*). It is likely that these differences in the α-limit dextrins are related to the concentration of enzyme used in their formation. Conditions conducive to the hydrolysis of maltotriose would seem also to facilitate a further hydrolysis of the α-limit dextrins that accumulate at the end of the first stage of the amylolysis. Amylolysates of amylopectin and glycogen at the limits of the first and second stages analysed by paper chromatography revealed patterns of sugar spots in agreement with the results of Roberts & Whelan (1960) and Nordin & French (1958) (Fig. 5).

It will be seen from Fig. 6 that the penta- and tetra-saccharide limit dextrins carry the same maltotriose base chain (the three glucose units terminating in the reducing-end group), but differ in the nature of the side chain. It may be that in the second stage of α-amylolysis the tetrasaccharide is formed from the pentasaccharide by a slow splitting of glucose from the maltose side chain. More likely it is formed from a hexasaccharide (6^3-α-maltotriosylmaltotriose), believed by Roberts & Whelan (1960) to be one of the first-stage α-limit dextrins and shown in Fig. 6*c*. The maltotriose side chain of this hexasaccharide, like free maltotriose, could perhaps be split to yield the tetrasaccharide and maltose.

The question may be posed whether the second stage of salivary α-amylolysis represents the final limit of its action. Glucose and maltose seem to be true end products, but the α-limit dextrins, with the tetrasaccharide (Fig. 6*a*) as the smallest of them, might still be degraded further. Meyer & Gonon (1951) contended that isomaltose was the

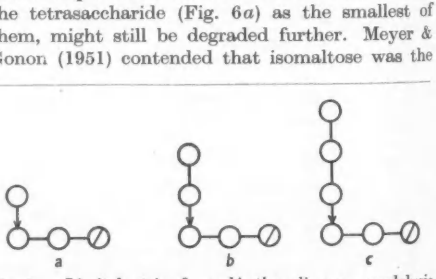

Fig. 6. α-Limit dextrins formed in the salivary α-amylolysis of amylopectin and glycogen. ○, Glucose unit; ⊘, reducing-end glucose unit; —, α-1:4-link; ↓, α-1:6-link.

end product of pig-pancreatic α-amylase action. Ahnström (1958) studied α-limit dextrins formed from Lintner starch by saliva and found the trisaccharide panose (4-α-isomaltosylglucose) as the smallest. The tetrasaccharide fraction was a mixture of possibly three substances. The conditions of the experiment were, however, such that it is uncertain whether the formation of panose represented a third stage of α-amylolysis. The digest contained a high concentration of enzyme [5 % (v/v) of filtered saliva] and was incubated for 3 months. Analysis of glucose and maltose after this time indicated that 'a rather strong hydrolysis of maltose to glucose must have taken place in the reaction mixture, i.e. the sample of saliva used contained maltase' (Ahnström, 1958). The same maltase, acting on α-limit dextrins, might have produced the panose.

SUMMARY

1. The actions of crude saliva and crystalline salivary α-amylase on starch and maltodextrins were compared, with particular reference to the question whether the α-amylase hydrolyses maltotriose. It is concluded that salivary α-amylase hydrolyses maltotriose to maltose and glucose. The hydrolysis is activated by chloride ion and is far slower than that of maltotetraose.

2. By varying the enzyme concentration the salivary α-amylolysis of amylose can be made to proceed in two stages. The first stage, at a low enzyme concentration, is a hydrolysis to maltose and maltotriose. The second stage, with enzyme at 25 times the concentration of that in the first stage, involves the hydrolysis of the maltotriose to glucose and maltose.

3. The α-amylolysis of amylopectin and glycogen also proceeds in two stages. The first-stage products are maltose, maltotriose and branched α-limit dextrins, ranging from a pentasaccharide upwards. The second-stage products are glucose, maltose and α-limit dextrins, of which the smallest is a tetrasaccharide.

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The Mechanism of Carbohydrase Action

8. STRUCTURES OF THE MUSCLE-PHOSPHORYLASE LIMIT DEXTRINS OF GLYCOGEN AND AMYLOPECTIN

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Cori & Larner (1951) described the preparation from rabbit muscle of an enzyme splitting the α-1:6-bonds of glycogen and amylopectin. This enzyme, amylo-1:6-glucosidase, did not act on the native polysaccharides but on the muscle-phosphorylase limit dextrins (φ-dextrins), from which glucose was liberated, the dextrins then becoming susceptible to further phosphorolysis. They concluded that in the φ-dextrin the side chains (*A* chains; Peat, Whelan & Thomas, 1952) were reduced in length to single glucose units, and that the outer portions of the main chains (*B* chains) were five to six units long, an estimate which was revised to six or seven units by Larner, Illingworth, Cori & Cori (1952) (Fig. 1). The unequal attenuation of the *A* and *B* chains by muscle phosphorylase is in contrast with the effect of β-amylase, since the β-limit dextrin (β-dextrin) of amylopectin contains *A* and outer *B* chains of equal length (two or three units) (Peat, Whelan & Thomas, 1952; Summer & French, 1956). Accordingly the φ-dextrins of amylopectin and glycogen were reinvestigated to find a reason for these differences. The evidence suggests that the structure of the φ-dextrin proposed by Cori & Larner (1951) is incorrect, in that the *A* chain is not a single glucose unit and that there is no great disparity, if any, between the lengths of the attenuated *A* and outer *B* chains.

A preliminary account of this work has already been published (Walker & Whelan, 1959*a*).

MATERIALS AND METHODS

Substrates

Waxy-maize starch (amylopectin) was prepared from hand-sorted single-cross Tapicorn seed (Bear Hybrid Corn Co., Decatur, Ill., U.S.A.) as by Schoch (1957). Rabbit-liver glycogen was prepared as by Schlamovitz (1951). Oyster glycogen was a commercial specimen (British Drug Houses Ltd.). Some properties of these three polysaccharides are listed in Table 1.

Amylopectin φ- and β-dextrin were prepared by incubating enzyme and substrate, as indicated below, for 4 and 24 hr. respectively. The enzymes were inactivated (3 min. at 100°), protein was centrifuged away and the supernatant solutions were deionized with Bio-Deminrolit resin (The Permutit Co. Ltd.) in the carbonate form (Woolf, 1953) before being dialysed in cellophan under toluene against running distilled water at room temperature for 48 hr. Where necessary the non-diffusate was concentrated in a rotary vacuum evaporator.

Maltotriose was prepared as by Peat, Whelan & Kroll (1956). Maltose (T. Kerfoot Ltd., Vale of Bardsley, Lancs.) was purified on charcoal–Celite (Whelan, Bailey & Roberts, 1953) to remove a possible trisaccharide impurity. Glucose was AnalaR-grade.

The concentrations of oligo- and poly-saccharide solutions were estimated by acid hydrolysis to glucose (Pirt & Whelan, 1951).

Enzymes

R-Enzyme. The enzyme was prepared from broad beans as by Hobson, Whelan & Peat (1951), except that, at the stage of removal of other enzymes by their adsorption on starch, the suspension was shaken for 24 hr. rather than stirred. This resulted in a more consistent preparation.

Amylo-1:6-glucosidase. The method of Cori (1955) was used as far as step 4. The enzyme was active on amylopectin φ-dextrin.

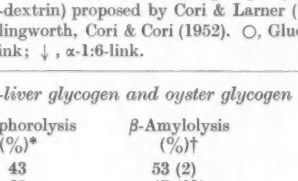

Fig. 1. Unit structure of muscle-phosphorylase limit dextrin (φ-dextrin) proposed by Cori & Larner (1951) and Larner, Illingworth, Cori & Cori (1952). ○, Glucose unit; —, α-1:4-link; ↓, α-1:6-link.

Table 1. *Properties of waxy-maize amylopectin, rabbit-liver glycogen and oyster glycogen*

	Average unit-chain length	Phosphorolysis (%)*	β-Amylolysis (%)†
Waxy-maize starch	24	43	53 (2)
Rabbit-liver glycogen	14	28	47 (21)
Oyster glycogen	11	20	39 (47)

* Conversion into glucose 1-phosphate.
† Conversion into maltose. Figures in parentheses indicate the time (hr.) taken to reach limiting conversion.

α-Amylase. Human salivary α-amylase was isolated as by Fischer & Stein (1954) and crystallized three times.

β-Amylase. The twice-crystallized sweet-potato enzyme was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Phosphorylases a *and* b. Phosphorylase *b* was prepared from rabbit muscle as by Fischer, Krebs & Kent (1958) and crystallized four times. It was converted into phosphorylase *a* (Fischer *et al.* 1958), which was also crystallized four times.

Enzyme assays

α-Amylase. The method of Schwimmer (1947) and Schwimmer & Balls (1949) for malt α-amylase was adapted for salivary α-amylase as by Walker & Whelan (1960).

β-Amylase. The activity was measured as by Hobson, Whelan & Peat (1950). Some difficulty was encountered with this enzyme owing to its instability. The suspension of crystals in ammonium sulphate supplied by the manufacturer (see above) was stable for several months in the refrigerator. Solutions made by diluting 0·1 ml. of suspension with 10 ml. of water (β_1 solution, 4000 units/ml.) lost about one-third of their activity in 1 day at 0°, and more dilute solutions (β_2 solution, 0·1 ml. of β_1 solutions with 1·9 ml. of water, 200 units/ml.) lost all activity in 1 hr. at 35°. This loss of activity was prevented by the addition of glutathione to 0·5 mM final concentration (Englard, Sorof & Singer, 1951). The solution then lost little activity during several days at 35°. When such solutions were diluted a further 20 times (β_3 solution, 10 units/ml.) the enzyme again became unstable, losing all activity in 4 hr. at 35°, and glutathione up to 5 mM-concentration was completely ineffective in preventing this. Freeze-dried human-serum albumin, in 0·05% concentration in β_3 solution, rendered the enzyme completely stable for 18 hr. at 35°. The albumin had no amylase activity. As a routine, therefore, 0·5 mM-glutathione was added to stock β_1 and β_2 solutions, which could then be stored for several weeks in the refrigerator. Serum albumin (0·05%) was added to all digests, irrespective of enzyme concentration, but no stabilizing agents were added to amylase-assay digests, for no loss of activity occurred during the 30 min. incubation period. In these digests the enzyme concentration is about 0·5 unit/ml. and the starch substrate presumably rendered the enzyme stable, for in the absence of starch there was an appreciable decay in activity in 30 min. The loss of activity in solution is not unexpected with low concentrations of a crystalline enzyme containing sulphydryl groups. On the other hand, in previous work with a several-times-crystallized enzyme prepared by Dr A. K. Balls (Balls, Walden & Thompson, 1948), it was not necessary to use stabilizing substances [see Table 3 in Peat, Pirt & Whelan (1952)].

Phosphorylase a. The method of Cori, Illingworth & Keller (1955) was used, except that adenylic acid was not added to the digest. Phosphorylase *a* prepared as by Fischer *et al.* (1958) is not activated by adenylic acid, in contrast with phosphorylase *a* prepared as by Cori *et al.* (1955).

Enzyme–substrate digestions

All digestions were at 35°, except where otherwise stated. Digests incubated overnight or longer were covered with toluene. Substrate-free controls were used to determine the reducing powers of the non-carbohydrate components. Enzymes were inactivated by heating for 3 min. at 100°. Reducing power was estimated with the Shaffer & Hartmann (1921) copper reagent. Glucose (60 mg./l.) was added to the reagent to compensate for loss of cuprous oxide by re-oxidation.

Action of R-enzyme. The digests contained 0·16% of polysaccharide, 55 mM-citric acid–NaOH buffer (pH 6·5) and R-enzyme (18 mg./ml.). For chromatographic examination the reaction was stopped after 3 hr. For determination of the increase in degree of β-amylolysis of the polysaccharide after debranching, the reaction was stopped after 24 hr. In control experiments with amylopectin under these conditions the increase in intensity of iodine stain consequent on R-enzyme action (Hobson *et al.* 1951) was complete after 24 hr. and no further change took place in the subsequent 24 hr. The extinction (680 mμ) of the stain at 24 hr. was 138% of its original value, having been 122% at 3 hr. Similarly, the degree of β-amylolysis of amylopectin β-dextrin rose from 0 to 98% after 22·5 hr. incubation with R-enzyme and remained at this value after 52·5 hr. When the debranching action was to be followed by β-amylolysis, the R-enzyme was inactivated and the digest adjusted to pH 5·5 with 0·2 M-sodium acetate–acetic acid buffer, pH 4·7.

*Action of amylo-*1:6*-glucosidase.* The enzyme solution (0·1 ml.) was incubated separately with maltotriose, maltose and glucose (10 mg., 0·5 ml.) for 24 hr. at room temperature. After the enzyme had been inactivated the digests were applied to paper for chromatography.

α-Amylolyses. Polysaccharides were hydrolysed with two concentrations of crystalline salivary α-amylase, as by Walker & Whelan (1960). The concentrations of the digest components were 0·4% of polysaccharide, 0·1 M-NaCl, 30 mM-citric acid–NaOH buffer (pH 6·5) and enzyme (0·25 or 0·0025 unit/ml.). The reducing power of the digests became constant after 48 hr.

β-Amylolyses. The digests contained 0·4% of polysaccharide, 0·5 mM-glutathione, 0·05% of serum albumin and 30 mM-sodium acetate–acetic acid buffer (pH 4·8). With debranched polysaccharide as substrate, the enzyme concentration was 10 units/ml. Digestion was complete within 4 hr. and hydrolysis of maltotriose during this time was negligible.

β-Amylolysis of native polysaccharides was carried out as described above but with 1000 units of enzyme/ml. The time taken to achieve limiting β-amylolysis varied with the type of polysaccharide, amylopectin or glycogen (see Table 1).

Phosphorolyses. The crystalline suspension of phosphorylase *a* in 30 mM-cysteine hydrochloride–40 mM-sodium glycerophosphate buffer (pH 6·8) was dissolved in the same buffer (0·2 ml. of suspension/10 ml. of buffer). The digestion mixtures contained 0·4% of polysaccharide, 80 mM-sodium phosphate (pH 6·8) and enzyme (520 units/ml.). The glucose 1-phosphate formed was freed from inorganic phosphate as by Liddle & Manners (1957) and estimated by hydrolysis as by Whelan & Bailey (1954), with Allen's (1940) colorimetric method. The degree of phosphorolysis attained a constant value at 4 hr., remaining unchanged during the subsequent 20 hr.

Paper chromatography

The paper was Whatman no. 3, previously washed in 1% acetic acid and distilled water. Descending chromatograms were irrigated with ethyl acetate–pyridine–water

(10:4:3, by vol.). The spray reagents were silver nitrate–NaOH (Trevelyan, Procter & Harrison, 1950) or aniline–diphenylamine–phosphoric acid (Harris & MacWilliam, 1954). Digests containing citrate buffer were deionized with Bio-Deminrolit resin (see above) before being applied to paper. Other digests were applied directly.

RESULTS AND DISCUSSION

Action of R-enzyme on φ-dextrin

R-Enzyme does not hydrolyse single α-glucose units joined through 1:6-links to starch molecules (Roberts, 1953; Whelan, 1953). The smallest side chain that can be detached is maltose. R-Enzyme should not therefore detach the *A* chains from φ-dextrin, if these are glucose units as postulated by Cori & Larner (1951). The only action of R-enzyme should be to split 1:6-links joining *B* chains to each other. Waxy-maize amylopectin φ-dextrin was treated with R-enzyme and then with β-amylase in order to estimate the extent of debranching. The yield of maltose from the Cori-type φ-dextrin should be 30%, according to calculations from the model φ-dextrin molecule constructed by Larner *et al.* (1952), assuming the average unit-chain length of the φ-dextrin to be 14. [The original amylopectin had chain length 24 and underwent 43% conversion into glucose 1-phosphate during phosphorolysis (Table 1).] The actual conversion into maltose after R-enzyme/β-amylase action was 79%, compared with 86% after the successive actions of R-enzyme and β-amylase on the original amylopectin and 92% from the β-limit dextrin (cf. Peat, Whelan & Thomas, 1956).

Action of α-amylase on φ-dextrin

When human salivary α-amylase attacks amylopectin and glycogen the reaction can be stopped at either of two stages, depending on the concentration of enzyme (Walker & Whelan, 1960). With a low concentration of enzyme the products are maltose, maltotriose and α-limit dextrins consisting of maltodextrins joined to each other through the α-1:6-branch links of the parent polysaccharide. The smallest of these α-limit dextrins is a pentasaccharide (Fig. 2*a*) (Bines & Whelan, 1960). With a 100-fold greater concentration of enzyme the maltotriose is hydrolysed to maltose and glucose and the α-limit dextrins are correspondingly attenuated, the smallest now being a tetrasaccharide (Nordin & French, 1958). This contains a glucose stub of the type postulated for φ-dextrin (Fig. 2*b*). If therefore these stubs are pre-existent in φ-dextrin, then the low concentration of α-amylase, which by itself cannot form the glucose stub, should nevertheless liberate the tetrasaccharide. The φ-dextrins of amylopectin, rabbit-liver glycogen and oyster glycogen were repeatedly examined by paper chromatography after α-amylolysis to this degree and the pentasaccharide was always the smallest α-limit dextrin (Fig. 3). When the φ-dextrins were treated with the high concentration of α-amylase the tetrasaccharide appeared (Fig. 3).

Fig. 2. α-Limit dextrins from glycogen and amylopectin. ○, Reducing glucose unit; other symbols are as identified in Fig. 1.

Products of debranched φ-dextrin

The treatment of amylopectin φ-dextrin with R-enzyme yielded substances behaving chromatographically as maltodextrins, the smallest being maltotetraose (Fig. 3). R-Enzyme would not, of

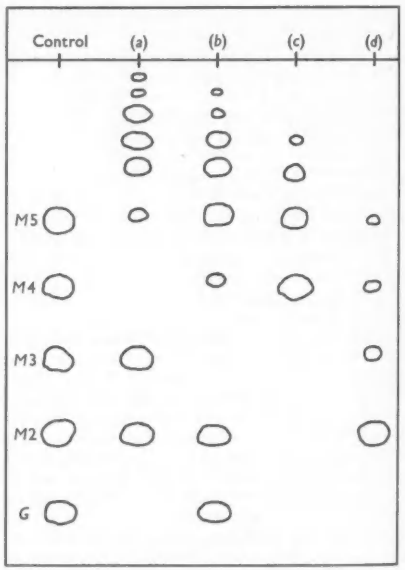

Fig. 3. (*a*) Paper-chromatographic fractionation of the products of action of dilute salivary α-amylase on the φ-dextrins of amylopectin, rabbit-liver and oyster glycogens. (*b*) Products of the action of concentrated α-amylase on the same three substrates. (*c*) Products of the action of R-enzyme on amylopectin φ-dextrin. (*d*) Products of the action of R-enzyme on amylopectin φβ-dextrin. Control substances are glucose (*G*), maltose (*M* 2), maltotriose (*M* 3), etc.

course, liberate glucose stubs if these were present (see above), but since the parent amylopectin with R-enzyme yielded no low-molecular-weight maltodextrins, the maltodextrins formed from the ϕ-dextrin were not preformed in the starch and clearly represented the residues left by phosphorolytic degradation. The smallest maltodextrin formed from the Cori ϕ-dextrin could not be smaller than maltoheptaose (Fig. 1). The ϕ-dextrin was treated with β-amylase and freed from maltose. The resulting $\phi\beta$-dextrin treated with R-enzyme now yielded maltose as a major component, with somewhat smaller amounts of maltotriose and higher maltodextrins (Fig. 3).

Structure of ϕ-dextrin

The foregoing evidence is consistent with an *A*-chain residue of the ϕ-dextrin at least four units long, and two units long in the $\phi\beta$-dextrin. It does not give direct information about the length of the outer portion of the *B* chain but this can be deduced from the action of β-amylase on the ϕ-dextrin. Hestrin (1949) found that about 1 mol.-prop. of maltose was liberated by β-amylase per unit chain of the ϕ-dextrin. Cori & Larner (1951) concluded that since the *A* chains were single glucose units the maltose must arise solely from the *B* chains and, assuming equal numbers of *A* and *B* chains, in the proportion of 2 mol.prop./chain. The *A* chain in the new formula (Fig. 4) would be attacked by β-amylase, losing 1 mol.prop. of maltose. The *B* chain must therefore be shorter than was supposed and could be four units long, losing 1 mol.prop. of maltose on β-amylolysis. The attenuation of the *A* chain and the outer *B* chain to four units by muscle phosphorylase is analogous to the similar attenuation of linear chains by the potato enzyme (Whelan & Bailey, 1954). Fig. 4 is a tentative picture of the average unit structure of glycogen and amylopectin muscle-phosphorylase limit dextrins. Further evidence for the picture is being sought. If it is correct then the calculation of the degree of multiple branching of amylopectin and glycogen from the degrees of phosphorolysis and β-amylolysis, as suggested by Liddle & Manners (1957), is not possible.

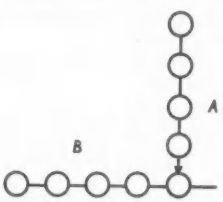

Fig. 4. Unit structure of muscle-phosphorylase limit dextrin proposed by the present authors. Symbols are as identified in Fig. 1.

Debranching action of amylo-1:6-glucosidase

If there are no glucose stubs in ϕ-dextrin then it is not readily apparent why amylo-1:6-glucosidase liberates glucose from the dextrin. An explanation advanced by Whelan (1958) requires that the preparations of amylo-1:6-glucosidase from rabbit muscle are contaminated with a starch-disproportionating enzyme of the type already known to exist in the potato (Peat, Whelan & Rees, 1953, 1956). This is D-enzyme, which is believed to be implicated in potato-starch metabolism (Walker & Whelan, 1959*b*). A similar enzyme is known in rat liver (Giri, Nagabhushanam, Nigam & Belavadi, 1955; Petrova, 1958; Stetten, 1959). D-Enzyme reversibly transfers segments of starch chains to other chains or to acceptors such as glucose and maltose (Peat, Whelan & Jones, 1957). Whelan (1958) suggested that a rabbit D-enzyme could act reversibly on the true ϕ-dextrin (Fig. 4) and convert it into the Cori–Larner molecule (Fig. 1). Only then could the glucosidase split off glucose. Other structures would also be formed by the D-enzyme, but the glucosidase could act only in the particular case where a glucose stub is exposed.

This hypothesis was examined as follows. Amylo-1:6-glucosidase from rabbit muscle was allowed to act on glucose, maltose and maltotriose. It had no action on glucose but maltose was converted into substances migrating on paper with glucose and maltotriose, and maltotriose into glucose, maltose, maltotetraose and higher oligosaccharides.

Support for this explanation of the action of amylo-1:6-glucosidase comes from the observation by Cori & Larner (1951) of a slow action of the enzyme on amylopectin β-limit dextrin. This dextrin contains no glucose stubs of the type postulated in ϕ-dextrin, and the *A* chains are two or three units long (see above). Therefore the glucosidase should have no action. That it has an action is at variance with the Cori–Larner hypothesis, but is explicable if the enzyme preparation contains a transferase capable of exposing glucose stubs.

The heterogeneity of the amylo-1:6-glucosidase preparation was further revealed in experiments on isomaltose carried out with Mr M. Abdullah. Cori & Larner (1951) reported that amylo-1:6-glucosidase attacks this sugar slowly and regarded the result as proving that the glucosidase attacks the 1:6-links of ϕ-dextrin. We purified the glucosidase as by Cori (1955) and the ammonium sulphate precipitate and supernatant solution at step 2 of the procedure was tested for hydrolytic activity towards isomaltose. The precipitate, said to contain 80% of the glucosidase (Cori, 1955), split

ϕ-dextrin. It was only very weakly active towards isomaltose, whereas the supernatant solution was strongly active. Even if pure amylo-1:6-glucosidase proves to have a slight isomaltose-splitting activity, there is certainly another enzyme in rabbit muscle with much more of this activity; indeed, an enzyme of this kind was found in rabbit muscle by Lukomskaia (1956). Moreover, the corresponding amylopectin-debranching enzyme of plants, R-enzyme, does not attack isomaltose (Hobson *et al.* 1951).

SUMMARY

1. The structures of the limit dextrins produced by the action of rabbit-muscle phosphorylase on glycogen and amylopectin were examined by enzymic methods. The formula previously assigned by Cori & Larner (1951) to the outer chains of the dextrins is incorrect. The side chains are not single glucose units but are probably four units long. Correspondingly the outer portions of the main chains are shorter than was supposed.

2. A preparation of amylo-1:6-glucosidase, the enzyme which splits the branch links in the phosphorylase limit dextrins, proved to be contaminated with a transglycosylase, acting on maltodextrins. The presence of this second enzyme may account for the formation of glucose when the amylo-1:6-glucosidase acts on phosphorylase limit dextrins.

3. The splitting of isomaltose by crude muscle extracts is largely, if not entirely, mediated by an enzyme distinct from amylo-1:6-glucosidase.

4. The instability of weak solutions of crystalline β-amylase was investigated and overcome by the addition of glutathione and serum albumin.

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Properties of the Ultraviolet-Absorbing Lipids Produced by Rat Adrenals *in vitro*

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In their publication on the microestimation of steroids produced by rat adrenals *in vitro*, Elliott, Birmingham, Schally & Schonbaum (1954) pointed out that consistently higher values were obtained when the steroids were measured by ultraviolet absorption (corrected for the effect of interfering substances), than by reduction of a tetrazolium derivative. Addition of adrenocorticotrophic hormone to the medium increased the steroid values obtained by both methods as well as the absolute difference between the values. They concluded that, in addition to known corticoids, the isolated rat adrenal produces material with an α:β-unsaturated ketone group but without a reducing α-ketol side chain, and that the formation of this material was stimulated by adrenocorticotrophic hormone. More recently it was shown that a large proportion of the ultraviolet-absorbing lipids produced by the rat adrenal is located in the zone called X by Heard *et al.* (1954), after chromatography in toluene–propylene glycol, and that this material does not reduce tetrazolium but gives a typical Porter–Silber spectrum (Birmingham & Kurlents, 1959). It appears to be identical with the non-reducing Porter–Silber chromogen isolated from rat-adrenal-vein blood by Reif & Longwell (1958), with a mobility of 0·62 in the toluene–methanol–water (4:3:1, by vol.) system of Bush. A non-reducing ultraviolet-absorbing lipid with similar mobility had been found earlier in rat blood by Bush (1953) and by Vogt (1955).

Aside from zone X, three major ultraviolet-absorbing regions are obtained when lipids from incubated rat adrenals are chromatographed in toluene–propylene glycol. These have been designated by Heard *et al.* (1954) as zones F, E and B and have been associated with cortisol, cortisone–aldosterone, and with corticosterone and *epi*-corticosterone (Heard *et al.* 1954, 1956; Eisenstein, 1956). The production of appreciable amounts of material with mobility of 11-deoxycorticosterone has also been reported (Koritz, Péron & Dorfman, 1957; Roberts, 1957). In a recent study of the spectra obtained after exposure of eluates from zones F and E to the Porter–Silber reagent no evidence for the liberation of cortisol or cortisone by the rat adrenal could be obtained (Birmingham & Kurlents, 1959).

The present investigation deals with a closer characterization of the ultraviolet-absorbing lipids produced by isolated rat adrenals and with effects upon their elaboration of stimulation by adrenocorticotrophic hormone, of prolonged incubation and of variation in the substrates and ions of the medium. Parts of this work have been presented at the First Meeting of the Canadian Federation of Biological Sciences in June 1958 and at the 43rd Annual Meeting of the Federation of American Societies for Experimental Biology in April 1959.

METHODS

Incubation and chromatography

Adrenals from 8–30 male Sprague–Dawley rats, anaesthetized with sodium pentobarbital, were quartered and incubated at 38° in Ringer bicarbonate–glucose solution for 2–10 hr. with hourly changes of the medium. Adrenocorticotrophic hormone (ACTH; U.S.P. or Connaught lot no. 34) was used at concentrations of 100–200 milliunits/100 mg. of tissue. The methylene chloride extracts of the incubation media were chromatographed in the toluene–propylene glycol system of Burton, Zaffaroni & Keutmann (1951) on strips of Whatman no. 42 filter paper at 31° for 48–60 hr. For the estimation of lipids less polar than corticosterone the time of development was adjusted to prevent the solvent front from running off the paper, or the effluent was rechromatographed. Acetylated lipids were prepared and developed in the benzene–formamide system as outlined by Zaffaroni & Burton (1951).

Detection of steroids on paper

Ultraviolet-absorbing lipids were located by scanning the paper strips in the Beckman model DU spectrophotometer at 240 mμ. A diaphragm placed in the light path confined the width of the paper exposed to the ultraviolet light to 3 mm. Reducing lipids were made visible with an alkaline solution of the tetrazolium derivative M. and B. 1767 (May and Baker Ltd.) [2 mg. of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride + 2 ml. of 95% (v/v) ethanol + 5 ml. of 2·5 N-NaOH], Porter–Silber chromogens by passing the paper through the Porter–Silber reagent (Birmingham, 1959). With the latter procedure a bright-yellow colour developed immediately at room temperature with the 21-aldehydes of steroids, or in 0·5–2 hr. with steroids containing the dihydroxyacetone side chain. The following substances could be detected with this reagent in amounts of at least 2 μg./cm.^{2}: cortisol, cortisone, 11-deoxy-17-hydroxycorticosterone and their acetates; Schiff-positive, non-reducing oxidation products of cortisol, cortisone,

11-deoxy-17-hydroxycorticosterone, corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone and their acetates. With 50 μg. of corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone and 17-hydroxyprogesterone no colour was obtained.

Other tests on paper included the Zimmermann test and the reaction for 17:20-diols by oxidation with lead tetraacetate, followed by the Zimmermann test (Bush, 1955), the vanillin test (McAleer & Kozlowski, 1956), exposure to concentrated sulphuric acid (Zaffaroni & Burton, 1951) and the Schiff test (Birmingham, 1959). In the last-named procedure the paper strips were passed through a 1% solution of *p*-rosaniline hydrochloride in sulphurous acid. Freshly prepared 21-aldehydes of cortisol, cortisone, 11-deoxy-17-hydroxycorticosterone, corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone (10 μg./cm.^{2}) caused the development of a purple spot which appeared as the rest of the strip turned pink. The corresponding ketols did not react in amounts of 50 μg./cm.^{2}, nor did aldosterone. It was noted that freshly prepared aldehydes reacted at a lower concentration than material which had been stored in the cold in methanol, or which had been chromatographed. The aldehydes were prepared by a procedure adapted from that of Beyler & Hoffman (1957) for the preparation of 21-aldehydes of corticosterone and 11-dehydrocorticosterone. The steroid (10–20 mg.) was dissolved in 4 ml. of methanol. A 3% (w/v) solution of cupric acetate in 80% (v/v) methanol (1 ml.) and 1 drop of acetic acid were added and the mixture was heated in a water bath at 60°. Spot tests were performed at intervals on portions of 10 μl. Solutions containing originally 11-dehydrocorticosterone and 11-deoxycorticosterone gave negative reactions with tetrazolium after 15 min. Solutions of corticosterone, 11-deoxy-17-hydroxycorticosterone, cortisone and cortisol ceased to reduce after 30 min. The formation of a 21-aldehyde was assumed when the steroids spotted on paper gave a negative reaction with the tetrazolium derivative, a rapidly developing bright-yellow colour with the Porter–Silber reagent and a positive Schiff test.

Tests on eluted lipids

Analytical procedures on eluted lipids included ultraviolet-absorption measurements to define a peak at 240 mμ, reduction of the tetrazolium derivative M. and B. 1767, and the Porter–Silber reaction as outlined in previous publications (Elliott *et al.* 1954; Birmingham & Kurlents, 1958); the formation of dinitrophenylhydrazones (Weichselbaum & Margraf, 1955); formation of 17-oxo steroids before and after oxidation by sodium bismuthate (Bush & Willoughby, 1957) and by chromic acid (Wilson & Fairbanks, 1955); reaction with diphenylamine (Clark, 1955) and the determination of sulphuric acid spectra (Bernstein & Lenhard, 1953). For the last-named procedure a further purification of the eluates by application on a silica-gel column and gradient elution with benzene–ethyl acetate was sometimes necessary. Photometric readings were obtained in the beginning of this study with the Beckman model DU spectrophotometer and later with the DK-2 recording spectrophotometer. With microcuvettes, readings in 0·3 ml. of solution could be obtained.

RESULTS

Distribution of ultraviolet-absorbing lipids on the chromatogram

It is apparent from Table 1 that the relative amounts of lipid recovered from the various zones depend upon the state of the gland. Averages of between 2 and 4 μg./100 mg. of adrenal/2 hr. were obtained in the absence of ACTH from each of the four main zones, and around 1 μg. from a zone corresponding to the location of 11-dehydrocorticosterone (zone A). When adrenals were incubated with ACTH the amount of lipid recovered from zones X and B increased 3–11 times, whereas there was little effect on the contents of zones F, E and A. In addition, about 0·3–1 μg./100 mg. of adrenal/2 hr. of an ultraviolet-absorbing, reducing material with the mobility of 11-deoxycorticosterone in the toluene–propylene glycol system was obtained occasionally from the medium of adrenals incubated with ACTH (see also Fig. 3).

Table 1. *Ultraviolet-absorbing lipids produced by rat adrenals incubated with and without adrenocorticotrophic hormone, and separated by paper chromatography in toluene–propylene glycol*

Amounts given under 'average recovery' are based on ultraviolet absorption; the values have not been corrected for a loss of about 10% in the extraction of the incubation medium with methylene chloride and for losses during chromatography. The recovery after elution of 10 samples of standard cortisol averaged 78±12 (S.D.)%, and that of seven samples of corticosterone 87±13%. The combined amount of adrenal lipid eluted from zones F, E, X and B averaged 72±13% of the total ultraviolet-absorbing lipid applied, in 22 chromatograms. Figures given in parentheses represent numbers of experiments.

	Average recovery (μg./100 mg. fresh wt./2 hr.±S.E.)		Ratios of stimulated to unstimulated production by the same glands				
Zone	No ACTH	ACTH	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
F	3·2±0·74 (5)	3·7±0·31 (8)	1·3	1·3	1·7	—	1·7
						2·5 (F+E)	
E	2·8±0·42 (6)	3·1±0·34 (9)	1·4	1·1	1·7	—	1·1
X	2·6±0·58 (7)	10·6±0·89 (10)	3·5	3·3	7·9	5·2	11·1
B	4·0±0·48 (8)	20·4±1·69 (11)	3·4	4·6	9·1	6·4	11·4
A	0·9±0·20 (3)	1·6±0·21 (4)	1·2	—	—	1·7	2·0
DOC	0·0 (2)	0·6±0·33 (3)					
Total	13·5	40·0					

Identity of ultraviolet-absorbing lipids

Ultraviolet-absorption spectra. The eluates from zones F, X, B and DOC had absorption maxima near 240 mμ, those of zones E and A near 238 mμ. The eluate of the solvent front did not give a curve characteristic for a Δ^4-3-ketone and absorbed ultraviolet light maximally in the region below 225 mμ.

Reducing power and phenylhydrazone formation. The ratios of reducing lipid, and of Porter–Silber chromogen to ultraviolet-absorbing lipid, obtained with eluates from the different zones are given in Table 2. Expected ratios near unity of reducing lipid to ultraviolet-absorbing lipid, and of Porter–Silber chromogen to ultraviolet-absorbing lipid, were obtained with all chromatographed steroid standards containing respectively an α-ketol and an α-dihydroxyacetone side chain. Adrenal lipids eluted from zones E and B gave average ratios of reducing to ultraviolet-absorbing lipid near 0·8, those of zones F and X less than 0·2. The ratios of Porter–Silber chromogen to ultraviolet-absorbing lipid obtained from zones F, E and B were less than 0·3, those of zone X near 0·9. These data indicate that cortisol and cortisone account for little, if any, of the ultraviolet-absorbing lipid found in the two more polar zones and that the Porter–Silber chromogens are represented by the non-reducing lipid in zone X.

The dinitrophenylhydrazones of steroids in alkaline solution yield absorption spectra that

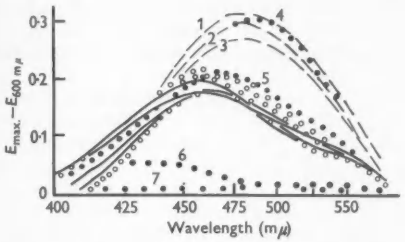

Fig. 1. Absorption spectra of the dinitrophenylhydrazones of adrenal lipids from zones F, E, X and B (solid lines); of the dinitrophenylhydrazones of corticosterone, 11-deoxycorticosterone, progesterone and 11-hydroxyprogesterone (○); of cortisol, 17-hydroxy-11-deoxycorticosterone and cortisone (broken lines, curves 1, 2 and 3); of 17-hydroxy-11-deoxycorticosterone-21-aldehyde, and of 11-dehydrocorticosterone (●, curves 4 and 5). Curves 6 and 7, spectra obtained with dehydro*iso*androsterone and cortolone. The dinitrophenylhydrazones were prepared by a modification of the procedure of Gornall & MacDonald (Weichselbaum & Margraf, 1955). Concentration of lipid, 10 μg./ml. (adrenal lipid based on ultraviolet absorption).

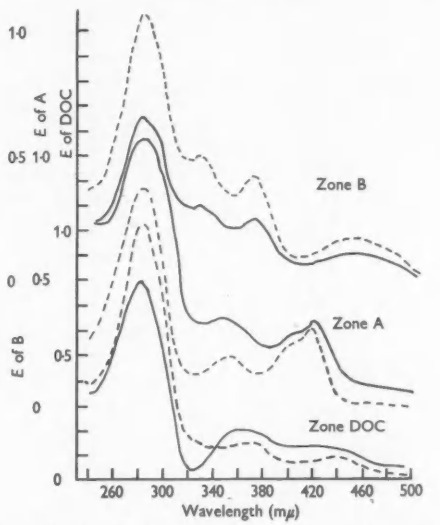

Fig. 2. Sulphuric acid spectra of rat-adrenal lipids eluted from zones B, A and DOC of the toluene–propylene glycol chromatogram and of chromatographed and eluted corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone. —, Adrenal lipids; - - -, reference steroids.

Table 2. *Ratios of reducing lipid and of Porter–Silber chromogen to ultraviolet-absorbing lipid obtained with extracts of incubation media of rat-adrenal glands and with reference steroids chromatographed in toluene–propylene glycol*

The reference steroids in zones F, E and B were cortisol, cortisone and corticosterone respectively. Results are given ± S.D. Figures in parentheses represent numbers of experiments.

	Reducing lipid/ultraviolet-absorbing lipid		Porter–Silber chromogen/ultraviolet-absorbing lipid	
Zone	Adrenal lipid	Reference steroid	Adrenal lipid	Reference steroid
F	0·19 ± 0·11 (3)	0·92 ± 0·02 (2)	0·04 ± 0·04 (2)	1·00 ± 0·00 (2)
E	0·87 ± 0·15 (3)	1·00 ± 0·17 (2)	0·18 ± 0·08 (2)	1·11 (1)
X	0·14 ± 0·06 (2)	—	0·86 ± 0·22 (6)	—
B	0·75 ± 0·14 (7)	0·86 (1)	0·28 (1)	—
A	0·86 (1)	—	—	—

depend to some extent upon the structure of the steroid (Gornall & MacDonald, 1953). The spectra obtained after reaction of dinitrophenylhydrazine for 90 min. at 59° with chromatographed eluted steroid standards and with adrenal lipids are shown in Fig. 1. Adrenal lipids from zones F, E, X and B showed absorption maxima near 460 mμ, as did standard corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, progesterone and 17α-hydroxyprogesterone. The maximum of cortisone, 11-deoxy-17-hydroxycorticosterone and cortisol on the other hand was at 480 mμ and the maximum absorption was higher than that of the steroids lacking a dihydroxyacetone side chain. Higher absorption values were also obtained with C-21 aldehydes of steroids containing a ketol or a dihydroxyacetone side chain, but with a maximum located near 490 mμ.

Sulphuric acid spectra. The sulphuric acid spectra of the lipids eluted from zones F and E show an absorption maximum near 280 mμ, but lack the pronounced maxima in the wavelengths above 400 obtained with cortisol and cortisone. The lipids in zones B, A and DOC gave similar spectra to those of corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone respectively (Fig. 2). The spectrum of the eluate from zone X had only one maximum, located at 290 mμ.

Reaction with diphenylamine. An absorption maximum at 530 mμ was not obtained with adrenal lipids from zones F, E, X and B exposed to diphenylamine, suggesting the absence of a ketone group at C-11 from the major lipid components of these zones.

17-*Oxo steroid formation.* No 17-oxo steroid could be detected in any region of the toluene–propylene glycol chromatogram including the solvent front (Fig. 3).

17-*Oxogenic steroids.* Ultraviolet-absorbing lipids eluted from zones F, E, X and B, and respotted in amounts of 20 μg. over an area of less than 1 cm.^{2}, failed to give the Zimmermann test after oxidation with lead tetra-acetate, whereas a strong positive reaction was obtained with 10 μg. of cortolone. Oxidation of the eluates from these zones with sodium bismuthate or chromic acid did not liberate a 17-oxo steroid.

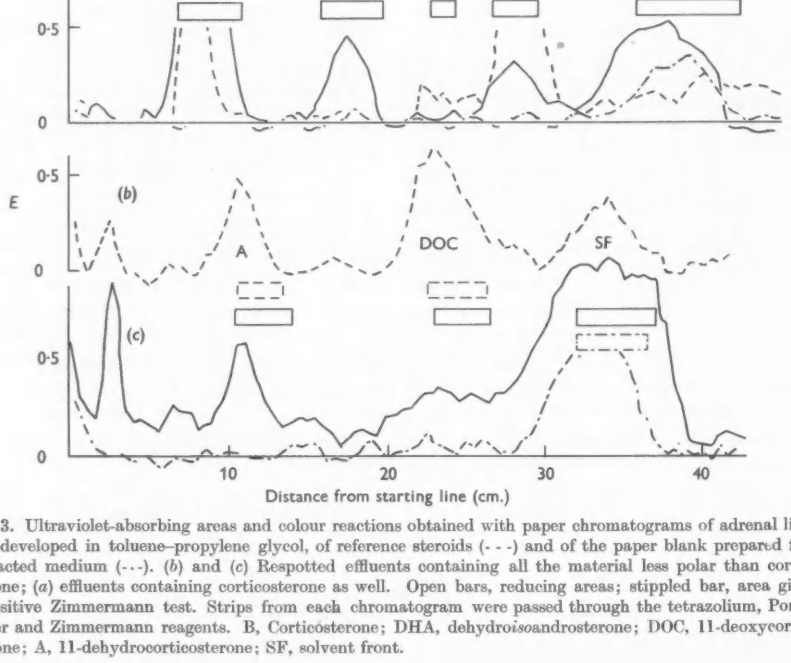

Fig. 3. Ultraviolet-absorbing areas and colour reactions obtained with paper chromatograms of adrenal lipids (—) developed in toluene–propylene glycol, of reference steroids (- - -) and of the paper blank prepared from extracted medium (···). (*b*) and (*c*) Respotted effluents containing all the material less polar than corticosterone; (*a*) effluents containing corticosterone as well. Open bars, reducing areas; stippled bar, area giving a positive Zimmermann test. Strips from each chromatogram were passed through the tetrazolium, Porter–Silber and Zimmermann reagents. B, Corticosterone; DHA, dehydro*iso*androsterone; DOC, 11-deoxycorticosterone; A, 11-dehydrocorticosterone; SF, solvent front.

Acetylation. The number and position of ultraviolet-absorbing regions obtained after acetylation at room temperature of lipids in zones F, E, X and B, and their colour reactions, are compared with those of acetylated reference steroids in Table 3. Zone F yielded two ultraviolet-absorbing, non-reducing regions considerably less polar in the benzene–formamide system than cortisol acetate. A major, ultraviolet-absorbing, reducing substance with R_F near 0·8 and a small non-reducing region close to the solvent front were obtained with lipid eluted from zone E. A mixture of 20 μg. of the acetylated lipid with R_F near 0·8 and 20 μg. of aldosterone diacetate did not separate on chromatography in benzene–formamide. Zone B yielded an acetate in the exact location of corticosterone acetate. When the Porter–Silber chromogen eluted from zone X was acetylated and chromatographed in benzene–formamide, two ultraviolet-absorbing substances giving a positive Porter–Silber reaction were obtained, one with R_F near 0·5, the other with R_F near 0·9. Mixed chromatography indicated that the slow-moving material was slightly more polar than the acetate of 11-deoxy-17-hydroxycorticosterone, from which it could be distinguished by its lack of reducing power. It moved with the same speed as corticosterone acetate but differed from it by giving a positive Porter–Silber spot test and a negative reaction with tetrazolium. In agreement with the results of Reineke (1956), corticosterone acetate was found, upon mixed chromatography, to be more polar than 11-deoxy-17-hydroxycorticosterone acetate, reversing the order of mobility of the parent compounds.

Comparison with 21-aldehydes. The 21-aldehydes of corticosterone and 11-dehydrocorticosterone form phenylhydrazones with an absorption maximum at 410 mμ (Silber & Porter, 1957) and do not reduce tetrazolium (Simpson & Tait, 1953; Beyler & Hoffman, 1957). Several steroids containing an α-ketol side chain were therefore oxidized with cupric acetate in methanol after the procedure adapted from Beyler & Hoffman (1957). The mobilities in toluene–propylene glycol of the substances contained in the methanol solution after oxidation of the different steroids with cupric acetate, and their reaction with tetrazolium and with the Porter–Silber reagent, are indicated in Table 3. The main non-reducing products obtained after oxidation of cortisol and cortisone and giving the Porter–Silber reaction had mobilities similar to the parent compounds. The chromogen derived from corticosterone moved with one-third of the speed of corticosterone. The oxidation of 11-deoxy-17-hydroxycorticosterone resulted in a non-reducing Schiff-positive Porter–Silber chromogen with the exact mobility of the non-reducing Porter–Silber-positive lipid secreted by the rat adrenal. With all reference steroids, additional non-reducing substances were obtained that did not, however, except cortisol, give the Porter–Silber reaction. Their mobilities are indicated in Table 3.

When the oxidation product of 11-deoxy-17-hydroxycorticosterone was acetylated with excess of acetic anhydride at room temperature and chromatographed in benzene–formamide, a phenylhydrazone was found with the same mobility as the rapidly moving component obtained after acetylation of the adrenal chromogen. The Porter–Silber-positive acetylation products of the 21-aldehydes of 11-dehydro- and of 11-deoxy-corticosterone were also located near the solvent front. None of the oxidized steroids examined gave a Porter–Silber-positive acetylation product with R_F near 0·5.

Although the oxidation product of 11-deoxy-17-hydroxycorticosterone had the same mobility as the adrenal Porter–Silber chromogen in the toluene–propylene glycol system and gave a positive spot test with phenylhydrazine, examination of the rate of formation of the phenylhydrazone from eluted material and various other tests revealed distinct differences between the two compounds. The adrenal lipid as well as the two derivatives of different mobilities obtained after acetylation formed the phenylhydrazone slowly and at the same rate as cortisol and cortisone. The oxidation product of 11-deoxy-17-hydroxycorticosterone, on the other hand, developed an absorption maximum well within 1 hr., located at 425 rather than 410 mμ. The absorption was only about one-third of that obtained with the phenylhydrazone prepared from comparable amounts of the adrenal lipid, of cortisol and of the rapidly reacting 21-aldehyde of corticosterone. In several experiments the derivative of 11-deoxy-17-hydroxycorticosterone ceased to absorb after 24–48 hr. The dinitrophenylhydrazone of the adrenal lipid in alkaline solution had an absorption maximum near 460 mμ, that of 11-deoxy-17-hydroxycorticosterone-21-aldehyde at 490 mμ (Fig. 1).

After exposure to concentrated sulphuric acid 20 μg. of the derivative of 11-deoxy-17-hydroxycorticosterone became orange–brown, whereas a bright-yellow colour was obtained with the adrenal lipid as well as with 20 μg. of corticosterone and of cortisol. The characteristic red spot was given by 11-deoxy-17-hydroxycorticosterone. Cortisol, 11-dehydrocorticosterone and 11-dehydrocorticosterone-21-aldehyde remained colourless, corticosterone-21-aldehyde became yellow–brown. The oxidation product of 11-deoxy-17-hydroxycorticosterone gave a sulphuric acid spectrum with a maximum near 290 mμ and minor peaks at 345 and 460 mμ. The spectrum of the adrenal lipid and of both acetylated derivatives had only one maximum, located near 290 mμ.

In the vanillin spot test the adrenal lipid from zone X (20 μg./cm.²) gave a bright-yellow colour, whereas both 11-deoxy-17-hydroxycorticosterone and its aldehyde became wine-red. A pale yellow was obtained with cortisol, cortolone and 11-deoxycorticosterone, a dark brown with corticosterone-21-aldehyde. Corticosterone, 11-dehydrocorticosterone, cortisone, progesterone and 11β-hydroxyprogesterone remained colourless, whereas 17α-hydroxyprogesterone gave an orange colour, reported by McAleer & Kozlowski (1956) to be typical for 17-hydroxy-20-oxo-21-methylsteroids.

A positive Schiff reaction was never obtained with adrenal lipids.

Bioassays. Lipids eluted from zones X and B were examined for their ability to depress the number of eosinophils in adrenalectomized mice, as an index of their effect on carbohydrate metabolism. In the Speirs–Meyer (1951) assay 25 μg. of eluate from zone B caused a fall of 81% in eosinophils, whereas the same dose of lipid from zone X was completely inactive. A fall of 77% was obtained with 8 μg. of cortisone.

The effect of lipids eluted from zones E, X and B on the ratio of urinary sodium to potassium was measured by the isotope method of Simpson & Tait (1952), eight rats at each of three doses of standard 11-deoxycorticosterone acetate and eight rats at one dose of adrenal lipid being used. The material from zone E, assayed at a dose of 0·1 μg./rat, had an effect comparable with that of 3 μg. of 11-deoxycorticosterone acetate. The lipid from zone B was inactive at a dose of 5 μg./rat. Equivocal results were obtained with the eluate from zone X. In

Table 3. *Mobilities, reducing power and phenylhydrazone formation by rat-adrenal lipids and their derivatives and by reference steroids*

r, Reducing; p, positive Porter–Silber test. Mobilities relative to the solvent front, or to corticosterone (B), are given with the standard error and the number of observations where it exceeds one. Values given in bold type indicate location of the major ultraviolet-absorbing area. Acetylated products were developed in the benzene–formamide system, all others in toluene–propylene glycol. Reference steroids in zones: F, cortisol; E, cortisone (except those marked with an asterisk); S, 11-deoxy-17-hydroxycorticosterone; B, corticosterone; A, 11-dehydrocorticosterone; DOC, 11-deoxycorticosterone. The preparation of oxidation products and acetylated derivatives is described in the text.

	Zone F	Zone E	Zone X	Zone S
Adrenal lipid	B0·09±0·009(18)	B0·28±0·018(18)r	B0·59±0·014(21)p	—
Reference steroid	B0·09±0·006(19)rp	B0·24±0·006(19)rp B0·30r*	—	B0·77±0·011(6)rp
Reference steroid oxidized with Cu^{2+} ions	**B0·11**±0·01(2)p B0·39±0·00(2)p B1·25±0·05(2)	B0·30±0·00(2)p B1·15±0·05(2)	—	**B0·61**±0·016(10)p B1·40±0·00(2)
Acetylated adrenal lipid	0·64 0·90	**0·76r** 0·90	0·53±0·023(9)p 0·87±0·019(8)p	—
Acetylated reference steroid	**0·07**±0·03(2)rp 0·23rp	0·28±0·018(4)rp 0·70±0·076(4)r*	—	0·62±0·053(7)rp
Reference steroid oxidized with Cu^{2+} ions and acetylated	—	0·19r **0·31**p	—	0·49 **0·89**±0·021(4)p

	Zone B	Zone A	Zone DOC
Adrenal lipid	B0·98±0·024(17)r	0·32±0·019(5)r	0·63±0·026(3)r
Reference steroid	(B1·0)r	0·31±0·009(4)r	0·67±0·013(5)r
Reference steroid oxidized with Cu^{2+} ions	B0·05±0·00(3) B0·18±0·012(10) **B0·33**±0·021(17)p B0·95±0·029(4) B1·20	B0·35±0·00(2) B0·74±0·012(4)p	B0·80 **0·30**p
Acetylated adrenal lipid	0·62r	—	—
Acetylated reference steroid	0·57±0·029(5)r	0·60r	0·84±0·033(5)r
Reference steroid oxidized with Cu^{2+} ions and acetylated	0·26p	0·55 0·89p	0·78p

* Aldosterone and derivative.

three assays, at doses of 2·5, 5 and 5 μg./rat, effects corresponding to 1·9, 2·2 and 0·5 μg. respectively of 11-deoxycorticosterone acetate were obtained.

Time course

The adrenal produces ultraviolet-absorbing lipids at a constant rate for at least 4–5 hr. endogenously or in response to added ACTH, provided that the medium is renewed frequently to prevent accumulation of steroids and inactivation of ACTH (Saffran & Bayliss, 1953; Birmingham & Kurlents, 1958). The rate of production over a 10 hr. period of each major lipid fraction in the presence of ACTH is shown in Fig. 4. After 4–6 hr. the rate declines but the percentages of the different fractions remain constant and are characteristic for the stimulated gland.

Effects of calcium and of carbohydrate substrates

Calcium and glucose are required for the response of the rat adrenal to added ACTH (Birmingham, Elliott & Valère, 1953; Péron & Koritz, 1958; Schonbaum, Birmingham & Saffran, 1956). Neither calcium nor glucose is effective alone (Table 4).

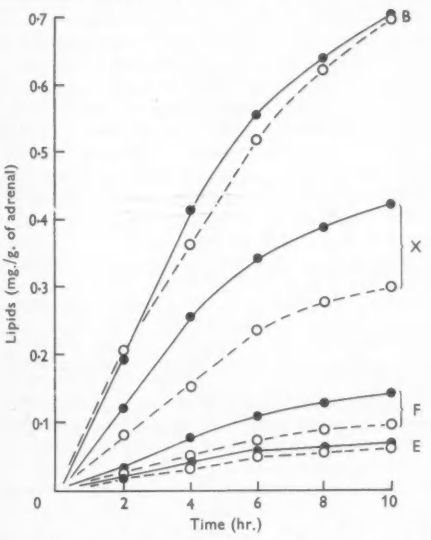

Fig. 4. Two experiments showing the time course of the production of ultraviolet-absorbing lipids of different mobilities by rat adrenals incubated with ACTH (200 milliunits/100 mg.). The medium was changed at hourly intervals and the production during 2 successive hours was pooled. Percentages of total eluate recovered in zones F, E, X and B during the first 2-hr. period: 9, 5, 33, 53 and 7, 6, 24, 62; during the fifth period: 12, 4, 30, 54 and 7, 4, 21, 68.

Both substances increase principally the production of corticosterone and the Porter–Silber chromogen, as does inosine (Tables 4 and 5). Ribose had no effect on the response to ACTH, in contrast with a previously published result (Schonbaum *et al.* 1956). Similar to the findings with calcium and glucose, inosine did not increase the production of steroids significantly in the absence of added ACTH.

DISCUSSION

The data indicate that the rat adrenal produces corticosterone, 11-dehydrocorticosterone and aldosterone *in vitro*. That the two first-mentioned substances are the main components of the eluates obtained from zones *B* and *A* of the toluene–propylene glycol chromatogram is apparent from the typical spectra obtained with sulphuric acid and from the ratios of ultraviolet-absorbing to reducing lipid. The material eluted from zone E had the following properties in common with aldosterone: mobility in the toluene–propylene glycol system, mobility of the acetylated derivative in benzene–formamide, ultraviolet- and sulphuric acid-absorption spectra, the ratio of ultraviolet absorption to reducing power, a negative Porter–Silber reaction and biological activity in the bioassay of Simpson & Tait at a dose of 0·1 μg./rat. Although a definite conclusion from only one assay at one dose is not justified, the material appeared to have one-third of the activity of aldosterone. The region may therefore also contain reducing, ultraviolet-absorbing and Porter–Silber-negative material without biological activity. Haynes, Koritz & Péron (1959) have recently renamed this zone aldosterone and mentioned that no cortisone was found to contaminate the aldosterone isolated from this region.

The identity of the material in zone F is not known. The lipid presumably contains an α:β-unsaturated ketone group as it absorbs maximally at 240 mμ in the ultraviolet range and its sulphuric acid spectrum has a peak near 290 mμ. It does not seem to contain an α-ketol or α-dihydroxyacetone side chain as it does not reduce tetrazolium and does not give a Porter–Silber reaction. Mialhe–Voloss & Baulieu (1958) have reported the production of a Δ^4-3-ketone-20:21-diol with the mobility of cortisol by incubated rat adrenals and Southcott, Bandy, Newsom & Darrach (1956) obtained 20-α-dihydrocorticosterone (pregn-4-ene-11β:20α:21-triol-3-one) in this region from extracts of mouse blood. Neither 20-α- nor 20-β-dihydrocorticosterone appeared to be among the lipids elaborated by rat adrenals *in vitro*. The sulphuric acid spectra of both isomers, prepared by reduction of corticosterone and separated by chromatography for 7 days as described by Southcott *et al.*,

had two pronounced maxima, near 290 and 390 mμ as opposed to the spectrum obtained from the adrenal lipid. However, the failure of the material to form a 17-oxo steroid after treatment with oxidizing agents, and the relatively low polarity of its acetylated derivatives, are compatible with a 20:21-diol structure. The absorption spectrum obtained with the diphenylamine reagent suggests the absence of a ketone function at C-11.

The lipid in zone X also remains to be identified. Ultraviolet-absorption spectra in methanol and in sulphuric acid, lack of reducing power and the spectrum obtained with diphenylamine are compatible with a steroid containing a Δ^4-3-ketone and lacking a ketone at C-11 as well as a readily reacting α-ketol side chain. The lipid is not 17-oxogenic and therefore does not appear to contain adjacent oxygen functions at C-17 and C-20. Acetylation of the material yielded two derivatives and it is therefore possible that more than one component is located in this zone. However, as both derivatives appeared to have the properties of the parent lipid, one component may be represented with more than one site available for acetylation. Differences in

Table 4. *Effects of calcium and of glucose on the output, by rat adrenals, of lipids of different mobilities in the toluene–propylene glycol system*

Adrenals were pre-incubated for 30 min. in glucose- and calcium-free medium. For the experimental period all media contained ACTH (200 milliunits/100 mg. fresh wt.) and, where indicated, 2·5 mM-calcium and 10 mM-glucose. The tissue was incubated for four successive 1 hr. periods with hourly changes of medium. Two separate experiments are shown. In experiment (*a*) all values are based on ultraviolet absorption. In experiment (*b*) lipids in zone X were determined by the Porter–Silber method, in all other zones by reduction of the tetrazolium derivative M. and B. 1767. All figures are expressed as μg./100 mg. fresh wt./2 hr.

					Calcium effect		Glucose effect		
Column no....	1	2	3	4	No glucose (2–1)	With glucose (4–3)	No calcium (3–1)	With calcium (4–2)	Effect of calcium + glucose (4–1)
Calcium ...	−	+	−	+					
Glucose ...	−	−	+	+					
Zone									
F (*a*)	1·7	1·0	2·3	3·5	−0·7	1·2	0·6	2·5	1·8
E (*a*)	0·7	0·9	1·5	2·2	0·2	0·7	0·8	1·3	1·5
(*b*)	0·8	1·1	2·6	1·7	0·3	−0·9	1·8	0·6	0·9
X (*a*)	1·3	1·9	1·1	6·7	0·6	5·6	−0·2	4·8	5·4
(*b*)	4·5	5·7	1·7	12·3	1·2	10·6	−2·8	6·6	7·8
B (*a*)	4·3	6·0	5·3	20·4	1·7	15·1	1·0	14·4	16·1
(*b*)	—	9·9	5·8	25·2	—	19·4	—	15·3	—
A (*b*)	1·5	1·1	0·8	2·2	−0·4	1·4	−0·7	1·1	0·7
Total (*a*)	8·0	9·8	10·2	32·8	1·8	22·6	2·2	23·0	24·8

Table 5. *Effects of different substrates on the output of rat-adrenal lipids* in vitro

All values are expressed as μg./100 mg. fresh wt./2 hr.; figures in parentheses are values obtained with the Porter–Silber reaction; all other values are based on ultraviolet absorption. ACTH, 200 milliunits/100 mg. fresh wt. Glucose, ribose, inosine, 10 mM; calcium, when present, 2·5 mM. In Expt. 3 adrenals were quartered and pre-incubated in glucose- and calcium-free Ringer bicarbonate solution; in all other experiments adrenals were halved and pre-incubated in glucose-free Ringer bicarbonate solution.

As the variation in production by samples from the same tissue in the absence of ACTH was always less than 30% and usually less than 15% in medium of the same composition, the values obtained by ultraviolet absorption or by the Porter–Silber reaction in the presence of ACTH indicate a significant increase.

No ACTH	Expt. 1	Expt. 3	Expt. 4	Expt. 5	Expt. 6
No addition	21, 22	—	—	—	—
Inosine	22, 27	16 (6)	—	—	—
Inosine, no calcium	—	11 (3)	—	—	—
Glucose	—	16 (8)	—	—	—
Glucose, no calcium	—	9 (4)	—	—	—
With ACTH	**Expt. 2**				
No addition	38, 38	—	66	43	54
Inosine	50, 54	36 (19)	77	74	66
Inosine, no calcium	—	18 (9)	—	—	—
Glucose	—	31 (15)	87	83	78
Glucose, no calcium	—	19 (8)	—	—	—
Ribose	—	—	55	55	49

rate of phenylhydrazone formation, in the reaction with sulphuric acid and in other spot tests, make it unlikely that the 21-aldehyde of 11-deoxy-17-hydroxycorticosterone forms a part of the lipid in zone X even though other properties are similar. The negative vanillin test suggests the absence of a 17-hydroxy-20-oxo-21-methyl function. There is thus no evidence so far that the rat adrenal produces a steroid with a hydroxyl group on C-17.

The presence of 6β-hydroxy-11-deoxycorticosterone in zone X (Koritz & Péron, 1958) is unlikely as this compound is reducing, and it has a maximum near 345 mμ in sulphuric acid exceeding that near 290 mμ (Bernstein & Lenhard, 1954).

The Porter–Silber chromogen is present in adrenal-vein blood (Reif & Longwell, 1958). It may be excreted in an altered form as no Porter–Silber reaction has been obtained with extracts of rat urine (Silber & Porter, 1957). It is not extracted with alkali (unpublished observations; see also Reif & Longwell).

Factors affecting the production of corticosterone, such as ACTH, glucose, calcium, inosine and pantothenic acid (Longwell, Reif & Hansbury, 1958), affect the production of the Porter–Silber chromogen to the same extent. Whether the last-named substance is a precursor or derivative of corticosterone or whether it is elaborated independently remains to be determined. No evidence for an increased production of the chromogen by the addition of corticosterone, and vice versa, has been obtained so far (Birmingham & Kurlents, 1958, and unpublished observations).

A possible effect of this ACTH-dependent Porter–Silber chromogen on electrolyte metabolism is of interest. Liberation of this material at a higher than normal rate into the vein blood from enucleated regenerated adrenals *in situ* and its production in higher than normal amounts by regenerated adrenals incubated with [4-^{14}C]progesterone have been reported (Laplante, Chappel, Stachenko & Giroud, 1959).

The lipid eluted from zone DOC had the same mobility, ultraviolet-absorption spectrum in methanol and reducing power as 11-deoxycorticosterone, and a similar, if not very distinctive, sulphuric acid spectrum. The poor yield of the substance has precluded further attempts at identification. The lack of a pronounced absorption in sulphuric acid at 380 mμ rules out the presence of 11β-hydroxyandrost-4-ene-3:17-dione as does also the negative Zimmermann reaction. These results are similar to those obtained by Reif & Longwell (1958) with the lipids of rat-adrenal-vein blood that have R_F 0·86 in the toluene–methanol–water system of Bush. The relative amounts recovered by Longwell *et al.* (1958) from the various zones are also strikingly similar to those obtained in the present work with glands stimulated by addition of ACTH to the medium. This is in line with the conclusion that the secretions *in situ* are those of an already stressed gland (Bush, 1953; Vogt, 1957). Useful information on factors affecting the secretion of the resting gland and on the requirements for the response to ACTH may thus be obtained by the method *in vitro* even though the rate of production is only one-fifth to one-tenth of that of the adrenal gland *in situ*. One reason for this difference is the accumulation during incubation of steroids and possibly other interfering substances, and the loss of activity of added ACTH.

The failure to detect cortisol, cortisone or 11-deoxy-17-hydroxycorticosterone in these studies and the poor recovery of material with the properties of 11-deoxycorticosterone, differ from the finding by Roberts (1957) of substances with chromatographic, spectrophotometric and staining characteristics of cortisol, cortisone and 11-deoxy-17-hydroxycorticosterone, and of the secretion by unstimulated rat adrenals *in vitro* of nearly equal amounts of material with mobilities of corticosterone and 11-deoxycorticosterone.

Haynes *et al.* (1959) have shown that adenosine 3′:5′-monophosphate is more effective than ACTH in stimulating the steroid production by incubated rat adrenals and conclude that the main function of ACTH is the activation of phosphorylase, which, by accelerating breakdown of glycogen and subsequent C-1 oxidation, generates the reduced triphosphopyridine nucleotide required for steroid synthesis. The role of glucose remains, however, to be clarified. In the presence of hexokinase it should become accessible to C-1 oxidation, obviating the need for the activation of phosphorylase. Yet it cannot stimulate steroid production in the absence of ACTH, not even when oxidized triphosphopyridine nucleotide is added (Koritz & Péron, 1958). This might be explained by an effect of ACTH at the hexokinase stage. The inability of adrenals to respond to ACTH in the absence of glucose agrees with this interpretation, but the successful substitution of inosine or adenosine (Schonbaum, Davidson, Large & Casselman, 1959) for glucose does not. It would be of interest to know whether adenosine 3′: 5′-monophosphate acts in the absence of glucose or of inosine and calcium. Calcium, but not glucose, has recently been found to be required for the extra-adrenal lipolytic action of ACTH (Lopez, White & Engel, 1959).

SUMMARY

1. Ultraviolet-absorbing lipids produced by incubated rat adrenals were separated by paper chromatography in toluene–propylene glycol, and some physical, chemical and biological properties were examined.

2. Lipids with chromatographic mobility, absorption spectra in methanol and in sulphuric acid, and with the reducing power of corticosterone, aldosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone were recovered.

3. No evidence was found for the production of lipids with a dihydroxyacetone side chain, of 17-oxo steroids and of 17-oxogenic steroids.

4. A non-reducing Porter–Silber chromogen was recovered with a polarity slightly greater than that of 11-deoxy-17-hydroxycorticosterone and with maximum absorption in methanol at 240 mμ and in sulphuric acid at 290 mμ. Acetylation of this material yielded two components, with R_F values 0·5 and 0·9 in benzene–formamide; other properties were the same as those of the parent compound.

5. The lipid with the mobility of cortisol in toluene–propylene glycol had properties expected of a Δ^4-3-ketone, did not reduce tetrazolium and its spectrum in sulphuric acid differed from those of both isomers of pregn-4-ene-11β:(20):21-triol-3-one. Upon acetylation, products of low polarity in benzene–formamide were obtained.

6. A dose of 0·1 μg. of lipid with the mobility of aldosterone depressed the urinary ratio of sodium to potassium in the rat by the same amount as 3 μg. of deoxycorticosterone acetate. The Porter–Silber chromogen gave variable results and the lipid with the mobility of corticosterone was inactive. In the eosinopenia assay the Porter–Silber chromogen was inactive at a dose of 25 μg./rat. A similar dose of the lipid with the mobility of corticosterone was one-third as active as cortisone.

7. The production of the Porter–Silber chromogen and of the lipid with the properties of corticosterone was greatly enhanced by adrenocorticotrophic hormone and by factors increasing the response to adrenocorticotrophic hormone, such as calcium, inosine and glucose. The effect on the other lipids was small or absent.

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Biological Synthesis of L-Ascorbic Acid in Animal Tissues: Conversion of D-Glucuronolactone and L-Gulonolactone into L-Ascorbic acid

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The synthesis of L-ascorbic acid in animal tissues is believed (Horowitz & King, 1953*a*, *b*; Isherwood, Chen & Mapson, 1954; Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957, 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960*a*) to follow the sequence:

$$\text{D-Glucuronolactone} \xrightarrow{\text{reduction}} \text{L-gulonolactone} \xrightarrow{\text{oxidation}} \text{L-ascorbic acid.}$$

The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid has previously been reported to be located in the microsomal fractions of the liver homogenates of the rat and goat, and of kidney homogenates of the chick (Burns *et al.* 1956; Chatterjee *et al.* 1958*b*, *c*; Roy & Guha, 1958). The synthesis from D-glucuronolactone, irrespective of the species studied, is greatly stimulated in the presence of sodium or potassium cyanide but the conversion of L-gulonolactone into L-ascorbic acid does not require the presence of cyanide and is, in fact, inhibited by it (Chatterjee *et al.* 1958*c*). The function of cyanide therefore lies apparently in the reduction of D-glucuronolactone to L-gulonolactone and it probably acts by activating the thiol enzyme involved in this reduction (Chatterjee *et al.* 1958*c*).

The rate of conversion of D-glucuronolactone into L-ascorbic acid by the microsomes from any of the species mentioned above is maximum with 50 mM-cyanide, the rate of synthesis decreasing with decreasing cyanide concentration. With rat-liver and chick-kidney microsomes no appreciable synthesis is observed with 5 mM-potassium cyanide even when the time of incubation is prolonged from 1·5 to 3·0 hr. It has, however, been observed that the synthesis by rat-liver microsomes takes place also in the presence of 5 mM or even a lower concentration of cyanide if reduced diphosphopyridine nucleotide, reduced triphosphopyridine nucleotide, glutathione or a chelating agent such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl, 8-hydroxyquinoline or the boiled rat-liver supernatant is added to the system (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1958*a*). Substances such as reduced di- and tri-phosphopyridine nucleotide and glutathione probably function by stimulating the action of cyanide in reducing the thiol-enzyme for the conversion of D-glucuronolactone into L-gulonolactone, which constitutes the first phase of the reaction. The metal-binding agents such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline, and the boiled supernatant have been reported previously (Chatterjee *et al.* 1958*a*; Chatterjee *et al.* 1960*a*) to enhance the conversion of L-gulonolactone into L-ascorbic acid. It is likely therefore that these agents promote the formation of L-ascorbic acid from D-glucuronolactone by stimulating the second phase of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid.

Goat-liver microsomes behave somewhat differently from rat-liver and chick-kidney microsomes. Although the maximum conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes takes place with 50 mM-KCN, appreciable synthesis is also observed with 5 mM or an even lower concentration of cyanide if the time of incubation is prolonged from 1·5 hr. to 3·0 hr. This synthesis by the goat-liver microsomes is not further augmented by any of the aforesaid agents.

The lactone forms of the precursors, D-glucuronolactone and L-gulonolactone, have been found to be specifically active in the microsomal synthesis of

ascorbic acid; the sodium salts of the corresponding free acids are not acted on (Chatterjee *et al.* 1958*b*, *c*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*, *b*; Chatterjee *et al.* 1960*a*). Our observation on the specificity of the lactone structure has recently been confirmed by Kanfer, Burns & Ashwell (1959) as well as by Bublitz & Lehninger (1959). The latter have now withdrawn the former claim (Grollman & Lehninger, 1957) that it was the free acid and not the lactone that was specifically acted on in the biosynthesis of L-ascorbic acid.

It has already been reported that the oxidative conversion of L-gulonolactone into L-ascorbic acid by liver microsomes of the rat and goat is an aerobic process (Chatterjee *et al.* 1958*b*, *c*). It had been supposed by some workers (Grollman & Lehninger, 1957) that the intermediate was 3-keto-L-gulonic acid. We had, however, considered for several reasons that 2-keto-L-gulonolactone (L-*xylo*hexulonolactone) was the likely intermediate (Chatterjee *et al.* 1960*a*). Recently the products of oxidation have actually been identified as L-*xylo*hexulonolactone and hydrogen peroxide (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*c*; Chatterjee, Ghosh, Ghosh & Guha, 1959*d*). The possibility of L-*xylo*hexulonolactone being the intermediate in the oxidation of L-gulonolactone to L-ascorbic acid has also been suggested by Kanfer *et al.* (1959).

This paper describes a detailed investigation, covering some of the aspects mentioned above, into the mechanism of conversion of D-glucuronolactone and of L-gulonolactone into L-ascorbic acid by the liver microsomes of the rat and goat and the kidney microsomes of the chick as well as by soluble-enzyme preparations obtained from these microsomes.

METHODS

Preparation of nuclei, cell debris and mitochondria. The nuclei and cell-debris fraction was sedimented at 500 *g* and the mitochondrial fraction was sedimented at 8500 *g*, by the method of Schneider & Hogeboom (1950). Both the fractions were washed twice with iso-osmotic sucrose solution.

Preparation of microsomes and soluble supernatant. The microsomes and the soluble supernatant were prepared by the method described by Chatterjee *et al.* (1960*a*).

Preparation of a soluble enzyme. Goat-liver microsomes (10 ml., equivalent to 320 mg. of protein) were added during 2 min. to a solution (5 ml.) of sodium deoxycholate (250 mg.) in sodium phosphate buffer (0·2 M, pH 7·4) with constant stirring. After addition, the mixture was shaken vigorously for another 1–2 min., diluted to 35 ml. with 0·2 M-phosphate buffer and centrifuged at 100 000 *g* for 40 min. in a Spinco ultracentrifuge. The residue was discarded and the protein fraction of the supernatant precipitated at 30% saturation of ammonium sulphate (pH 7·0) was collected and dissolved in 0·1 M-phosphate buffer (10 ml.), pH 7·2. The enzyme solution was then dialysed for 1 hr. against 30 vol. of sodium phosphate buffer (0·01 M, pH 7·2) with constant stirring. After dialysis the enzyme solution was centrifuged at 10 000 *g* for 15 min. and the sedimented protein fraction was discarded. The supernatant (10–12 mg. of protein/ml.) containing the active enzyme was stored at −20°. The temperature throughout the procedure was kept at 0–2°.

Rat-liver microsomes as well as chick-kidney microsomes gave active soluble enzyme preparations by the same treatment except that in these cases the amount of deoxycholate used was half that used with goat-liver microsomes. This was done because the rat-liver microsomes and chick-kidney microsomes, obtained from a given weight of the tissues, were found to contain approximately half the amount of the protein in goat-liver microsomes obtained from the same weight of tissue, and the amount of deoxycholate needed was dependent on the protein content of the microsomes. The activity of the soluble-enzyme preparation from rat-liver microsomes or from chick-kidney microsomes was almost equal to that from goat-liver microsomes on the protein basis and was only about one half on the basis of the weight of the fresh tissues.

The boiled supernatant was prepared by the method already described (Chatterjee *et al.* 1960*a*).

Incubation media. Unless otherwise mentioned, the test system contains 20 mM-sodium phosphate buffer (pH 7·2), 10 mM-D-glucuronolactone, 0·25 ml. of the microsomal dispersion (equivalent to 250 mg. of wet liver or kidney), KCN (50 mM or less as indicated in the text). Total volume was 2·5 ml., incubated at 37° in air for 1·5 hr.

When L-gulonolactone (5 mM) was used as the substrate, cyanide was omitted from the incubation media.

Estimation of ascorbic acid. Ascorbic acid was identified and estimated by the methods described by Chatterjee *et al.* (1960*a*). In the presence of potassium ferricyanide ascorbic acid was estimated by Roe & Kuether's (1943) method and in the presence of cyanide Roe & Kuether's method was used after removal of cyanide by acidification with HCl (M) and evaporation to dryness under suction at 40° and taking up the residue with a known volume of water.

Identification and estimation of D-glucuronolactone and L-gulonolactone. D-Glucuronolactone and L-gulonolactone were identified by paper chromatography and estimated by an adaptation of the hydroxamic acid procedure described before (Chatterjee *et al.* 1960*a*).

Estimation of protein. Protein was estimated by the method already described (Chatterjee *et al.* 1960*a*).

Estimation of Hg^{2+} *ion.* Hg^{2+} ion was estimated colorimetrically with dithizone (Feigl, 1954). Hg^{2+} ion present in the samples of adenosine triphosphate was estimated by adding, with vigorous shaking, 1 ml. of a solution of 5 mg. of dithizone in 100 ml. of carbon tetrachloride to 1 ml. of a solution of adenosine triphosphate (25–50 mg.) that had been heated on a boiling-water bath with a drop of concentrated HCl for about a minute. The mixture was then diluted to 4 ml. with absolute ethanol and the colour produced was compared with that obtained with a standard solution of $HgCl_2$ treated similarly.

Preparation of the o-phenylenediamine derivative of L-xylohexulonate. L-*xylo*Hexulonolactone formed as an intermediate in the enzymic oxidation of L-gulonolactone to L-ascorbic acid was trapped in the system as L-*xylo*-hexulonate. The *o*-phenylenediamine derivative of L-*xylo*-hexulonate was then prepared as follows (Lanning & Cohen, 1951).

The soluble-enzyme preparation (2 ml.) was incubated at 37° in air for 30 min. with 10 mg. of L-gulonolactone in the presence of 50 mM-sodium phosphate buffer, pH 7·9, and 4 mM-KCN, the total volume being 2·5 ml. Potassium cyanide was added to prevent the oxidation of the synthesized ascorbic acid to the corresponding dehydro form, because the latter has also been found to give a condensation reaction with *o*-phenylenediamine which might interfere with the identification of the ketogulonate. It may be mentioned that the ene-diol form of ascorbic acid has not been found to give such condensation reaction under similar conditions. After incubation the mixture was deproteinized with 0·25 ml. of 30% metaphosphoric acid and filtered. The filtrate (1 ml.) was neutralized with 10% NaOH and to this was added 0·1 ml. of a solution of 1·5% *o*-phenylenediamine in 0·25 M-HCl. The mixture was heated on a boiling-water bath for 30 min. The *o*-phenylenediamine derivative thus produced was identified by the methods described below.

Identification of the o-*phenylenediamine derivative of* L-*xylohexulonate.* (*a*) Paper-chromatographic analysis. A portion (50 μl., equivalent to approx. 20–25 μg. of the L-*xylo*hexulonate) of the *o*-phenylenediamine derivative prepared as stated above was applied on paper (Whatman no. 1) for chromatographic separation with butan-1-ol–formic acid (1:1) as well as phenol–water (9:1) as the irrigating solvents. A parallel run was given with a portion of the *o*-phenylenediamine derivative prepared from an authentic sample of L-*xylo*hexulonate. After the run was over (4–6 hr.) the paper was air-dried and the spots were located by their typical blue fluorescence in the ultraviolet light. No spot could be detected by incubating L-gulonolactone with a boiled enzyme preparation. The R_F values with the butanol and the phenol solvents were 0·94 and 0·90 respectively. The spot obtained with the *o*-phenylenediamine derivative of dehydroascorbic acid (R_F 0·89, butanol solvent) was distinguished by a typical yellow fluorescence.

(*b*) Absorption-spectra analysis. The absorption-spectra analysis of the *o*-phenylenediamine derivative of the biosynthesized L-*xylo*hexulonate was carried out according to the method of Lanning & Cohen (1951) with a Beckman model DU spectrophotometer. About 25 μl. of the compound (equivalent to approx. 10–12 μg. of L-*xylo*hexulonate) was added to a 1 cm. cuvette containing glass-distilled water and the absorption spectra were analysed over the region 325–360 mμ, an authentic sample of *o*-phenylenediamine derivative of L-*xylo*hexulonate being kept in the reference cell.

Identification and estimation of hydrogen peroxide. Hydrogen peroxide formed as a product of microsomal oxidation of L-gulonolactone to L-ascorbic acid has been identified and estimated indirectly by the coupled oxidation of methanol to formaldehyde in the presence of catalase, formaldehyde being estimated by a modified method of Keilin & Hartree (1945).

MATERIALS

D-Glucuronolactone and sodium D-glucuronate were purchased from the California Foundation for Biochemical Research, California.

L-Gulonolactone, sodium L-gulonate and L-*xylo*hexulonate were prepared by the methods described before (Chatterjee *et al.* 1960*a*).

Adenosine triphosphate (ATP, crystalline), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and reduced diphosphopyridine nucleotide (DPNH) were obtained from the Schwarz Laboratories Inc. Reduced triphosphopyridine nucleotide (TPNH) was generated *in situ* from TPN (2 mM), glucose 6-phosphate (5 mM) and yeast glucose 6-phosphate dehydrogenase (1·5 units). Antimycin A was obtained through the courtesy of Kyowa Fermentation Ind. Co. Ltd., Tokyo, Japan.

RESULTS

Intracellular localization of the enzyme system converting D-*glucuronolactone into* L-*ascorbic acid.* The relative enzymic activities of the different subcellular fractions for the conversion of D-glucuronolactone into L-ascorbic acid are given in Table 1. The results (Table 1) show that the entire activity of the homogenates from the livers of the rat and goat and from the kidney of the chick is located in the microsomal fractions. The observed activity of the mitochondria is probably due to their contamination by microsomes since it has been found that the activity of the mitochondria decreases with washing.

Effect of cyanide on the rate of microsomal conversion of D-*glucuronolactone into* L-*ascorbic acid.* The rate of conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes has been found to be maximum with 50 mM-KCN, decreasing with decreasing cyanide concentration, and is not appreciable with 5 mM-KCN. But if the time of incubation is prolonged some synthesis is observed even with 5 mM-KCN (Fig. 1). Chick-kidney microsomes behave similarly. It has, however, been found that with goat-liver microsomes the conversion of D-glucuronolactone into L-ascorbic acid in the presence of 5 mM-KCN approaches that obtained with 50 mM-KCN if the time of incubation is prolonged (Fig. 1).

Effect of some other factors in the presence of cyanide on the microsomal conversion of D-*glucurono-*

Table 1. *Conversion of* D-*glucuronolactone into* L-*ascorbic acid by different subcellular fractions in the presence of* 50 mM-*potassium cyanide*

Each fraction represents 250 mg. of original liver or kidney. Other conditions were as stated in the Methods section.

Fraction	Ascorbic acid synthesized (μmole)		
	Goat liver	Rat liver	Chick kidney
Homogenate	0·55	0·35	0·40
Nuclei, cell debris	0	0	0
Mitochondria	0·10	0·06	0·06
Microsomes	1·20	0·6	0·65
Soluble supernatant	0	0	0

lactone into L-*ascorbic acid*. It has been found that though no appreciable synthesis of L-ascorbic acid from D-glucuronolactone by the rat-liver microsomes occurs in the presence of 5 mM-KCN, a marked synthesis takes place if DPNH or TPNH is added to the system (Table 2). The reduced pyridine nucleotides can be more efficiently replaced by some metal-binding agents, namely sodium pyrophosphate, $\alpha\alpha'$-dipyridyl, 8-hydroxyquinoline or by reduced glutathione (GSH). Addition of DPNH, TPNH or GSH to a system containing any of these chelating agents does not further enhance the synthesis. Some synthesis is also observed with sodium citrate, ATP or ADP, but not with adenosine monophosphate (AMP). Nicotinamide and antimycin A were ineffective. The results are given in Table 2. None of the aforesaid factors can, however, promote the synthesis in the presence of 50 mM-KCN. In the absence of cyanide no synthesis is observed in any of these cases. The behaviour of chick-kidney microsomes is similar. But none of the agents which stimulated the synthesis by rat-liver and chick-kidney microsomes has any influence on the synthesis by goat-liver microsomes.

Effect of boiled supernatant on the conversion of D-*glucuronolactone into* L-*ascorbic acid by the microsomes*. It has been observed that in a system containing 5 mM-KCN and a chelating agent, the chelating agent can be completely replaced by the boiled rat-liver supernatant for the conversion of D-glucuronolactone into L-ascorbic acid. The boiled supernatant from rat liver is not, however, specific; that obtained from goat liver, chick kidney and even guinea-pig liver is also equally active (Table 3). Like the chelating agent the boiled supernatant obtained from any of these species cannot, however, further enhance the synthesis stimulated by 50 mM-KCN, whereas in the absence of cyanide it is ineffective. Chick-kidney microsomes behave in a similar way to rat-liver microsomes in the presence of the boiled supernatant, which, however, has no influence on the synthesis by goat-liver microsomes.

Some properties of the boiled supernatant. The activity of the boiled supernatant is almost entirely lost if it is dialysed for 72 hr. against 30 vol. of 10 mM-sodium phosphate buffer, pH 7·2, treated with 4 % trichloroacetic acid, shaken with activated charcoal or heated at 100° with N-HCl for 30 min. or with N-NaOH for 10 min. The activity of the

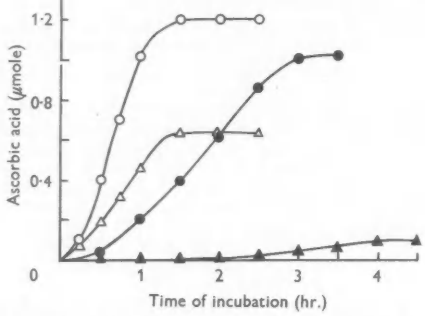


Fig. 1. Effect of cyanide on the rate of conversion of D-glucuronolactone into L-ascorbic acid by: ○, goat-liver microsomes with 50 mM-KCN; ●, goat-liver microsomes with 5 mM-KCN; △, rat-liver microsomes with 50 mM-KCN; ▲, rat-liver microsomes with 5 mM-KCN.

Table 2. *Effect of reduced pyridine nucleotides, chelating agents and some other factors on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by rat-liver microsomes*

Test system: where DPNH or TPNH was used, nicotinamide (30 mM) was added; incubation was for 3 hr. Other conditions were as stated in the Methods section. ADP, Adenosine diphosphate.

KCN (mM)	Factors	Concn. used (mM)	Ascorbic acid synthesized (μmole)
None	None	—	0
50	None	—	0·65
50	DPNH	1	0·66
50	Sodium pyrophosphate	5	0·67
5	None	—	0·02
5	TPNH	1	0·30
5	DPNH	1	0·25
5	Sodium pyrophosphate	5	0·75
5	$\alpha\alpha'$-Dipyridyl	2	0·70
5	8-Hydroxyquinoline	0·5	0·80
5	GSH	10	0·50
5	Sodium citrate	10	0·22
5	ATP	5	0·25
5	ADP	5	0·20
5	AMP	5	0·01
5	Antimycin A	1	0·01
5	Nicotinamide	30	0·02

Table 3. *Effect of boiled supernatant obtained from different species on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by rat-liver microsomes in the presence of* 5 mM-*potassium cyanide*

Test system: boiled supernatant, equivalent to 100 mg. of wet tissue; incubation was for 3 hr. Other conditions were as stated in the Methods section.

Source of boiled supernatant	Ascorbic acid synthesized (μmole)
None	Nil
Rat liver	0·80
Goat liver	0·50
Chick kidney	0·75
Guinea-pig liver	0·80

boiled supernatant is retained in the supernatant fraction after precipitating the protein with $Zn(OH)_2$ or by bringing down the pH to 3 with HCl. Attempts to separate the active factor present in the boiled supernatant by paper chromatography or paper electrophoresis have not given any clear-cut results.

Effect of L-*gulonolactone on the microsomal conversion of* D-*glucuronolactone into* L-*ascorbic acid.* It has been observed that though goat-liver microsomes do not convert D-glucuronolactone into L-ascorbic acid in the absence of cyanide, they do so when L-gulonolactone is added to the system (Tables 4 and 5). With an optimum concentration of L-gulonolactone (5 mM) the synthesis becomes maximum with an optimum concentration of D-glucuronolactone (10 mM), decreasing with the decreasing concentration of D-glucuronolactone (Table 4). Again with an optimum concentration of D-glucuronolactone (10 mM) the synthesis depends upon the concentration of L-gulonolactone present in the system, being maximum with the optimum concentration of L-gulonolactone (5 mM) and decreasing with its decreasing concentration (Table 5). The microsomes from rat liver or chick kidney behave similarly. The effect of L-gulonolactone on the conversion of D-glucuronolactone into L-ascorbic acid has been found to be similar to that obtained with KCN (Tables 4 and 5).

Effect of some electron acceptors on the conversion of D-*glucuronolactone into* L-*ascorbic acid by the rat-liver microsomes.* The effect of some electron acceptors on the synthesis of ascorbic acid has been studied. It has been found that although oxidized glutathione and methylene blue are ineffective, potassium ferricyanide at mM concentration completely inhibits the conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes in the presence of 5 mM-KCN and sodium pyrophosphate. Potassium ferricyanide, however, has no influence on the conversion of L-gulonolactone into L-ascorbic acid (Table 6).

Effect of adenosine triphosphate on the synthesis of ascorbic acid by rat-liver microsomes. Adenosine triphosphate has been found to stimulate the conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes in the presence of 5 mM-KCN (Table 2). It may be mentioned that in previous experiments (Chatterjee *et al.* 1958*c*) ATP was found to be inhibitory to the synthesis of ascorbic acid by rat- and goat-liver microsomes. But in subsequent investigations the observed inhibition by ATP was found to be due to the presence of Hg^{2+} ions (3 μg./mg.) in the sample of ATP used (Schwarz Laboratories Inc.; lot no. 5501, 1955) and not due to the ATP itself (Chatterjee *et al.* 1958*a*). Hg^{2+} ion has already been reported to be inhibitory to the synthesis of ascorbic acid (Chatterjee *et al.* 1958*c*, 1960*a*). Fig. 2 shows that the amount of Hg^{2+} ion present in the sample of ATP used can account for the observed inhibition. This shows, incidentally, that the biosynthetic reaction is very sensitive, even to traces of Hg^{2+} ion.

Effect of soluble supernatant on the relative conversion of D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid by the microsomes.* The microsomes in the presence of cyanide can convert D-glucuronolactone but not sodium glucuronate

Table 4. *Effects of* L-*gulonolactone and of potassium cyanide on the synthesis of* L-*ascorbic acid by goat-liver microsomes from different concentrations of* D-*glucuronolactone*

Concentrations of L-gulonolactone and potassium cyanide used were 5 and 50 mM respectively. Incubation was for 3 hr. The values of ascorbic acid given below with L-gulonolactone represent those obtained after deducting the value of ascorbic acid (1·40 μmole) obtained with L-gulonolactone alone.

D-Glucuronolactone (μmoles)	Ascorbic acid synthesized (μmole)	
	With L-gulonolactone	With KCN
50	1·20	1·10
25 (optimum)	1·30	1·20
12	0·62	0·55
6	0·35	0·30
3	0·18	0·16

Table 5. *Relative effects of different concentrations of* L-*gulonolactone and of potassium cyanide on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by goat-liver microsomes*

Concentration of D-glucuronolactone used was 10 mM. Other conditions were the same as stated in Table 4.

KCN (μmoles)	Ascorbic acid synthesized (μmole)	L-Gulonolactone (μmoles)	Ascorbic acid (μmoles) synthesized from	
			L-Gulonolactone	L-Gulonolactone plus D-glucurono-lactone
250	1·15	25	1·40	2·65
125	1·20	12	1·40	2·65
62	0·58	6	0·75	1·40
31	0·31	3	0·40	0·75
15	0·15	1·5	0·20	0·37

into L-ascorbic acid (Chatterjee *et al.* 1958*b*). The soluble supernatant, irrespective of species, when added to the system inhibits the synthesis from D-glucuronolactone (Fig. 3). The inhibitory factor in the supernatant has been traced to a lactone-splitting enzyme which hydrolyses D-glucuronolactone to D-glucuronic acid (identified by paper chromatography as the lactone after non-enzymic lactonization in the presence of HCl), the latter being not acted on by the microsomal enzyme. In the absence of cyanide, however, both the lactone and the sodium salt of the free acid are converted, though to a small extent, into L-ascorbic acid, provided that the soluble supernatant and TPNH are present along with the microsomes (Table 8). This synthesis in the presence of the soluble supernatant and TPNH is not much influenced by substrate concentration nor by the concentration of the soluble supernatant. On increasing the concen-

Table 6. *Effect of some electron acceptors on the synthesis of* L-*ascorbic acid by rat-liver microsomes*

The system contains 5 mM-sodium pyrophosphate and with D-glucuronolactone 5 mM-KCN was also present. Concentrations of potassium ferricyanide, methylene blue and oxidized glutathione were 1 mM, 1 μM and 2 mM respectively. Incubation was for 3 hr.

	Ascorbic acid synthesized (μmole)	
Electron acceptor	D-Glucurono-lactone	L-Gulono-lactone
None	0·80	1·30
Potassium ferricyanide	Nil	1·28
Methylene blue	0·80	1·30
Oxidized glutathione	0·78	1·25

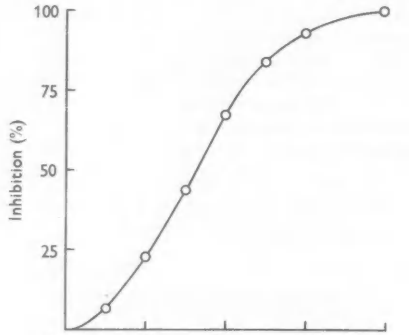


Fig. 2. Effect of Hg^{2+} ions on the synthesis of L-ascorbic acid from D-glucuronolactone by rat-liver microsomes. KCN (50 mM) was added 10 min. after the addition of $Hg(NO_3)_2$.

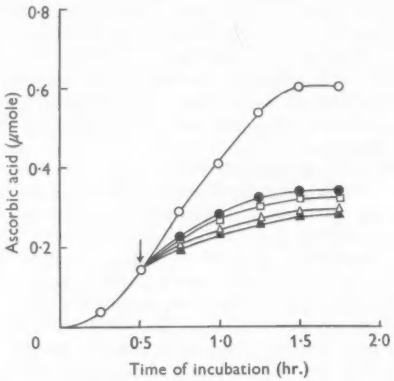


Fig. 3. Effect of soluble supernatants from organs of different species on the rate of conversion of D-glucuronolactone into L-ascorbic acid by rat-liver microsomes: ○, rat-liver microsomes without additions; ●, after addition of goat-liver supernatant; □, after addition of chick-kidney supernatant; △, after addition of guinea-pig-liver supernatant; ▲, after addition of rat-liver supernatant. The arrow indicates addition of 0·5 ml. of the supernatant.

Table 7. *Effect of varying concentrations of substrate, soluble supernatant and microsomes on the synthesis of* L-*ascorbic acid from* D-*glucuronolactone and sodium glucuronate*

Test system: TPNH, mM; nicotinamide, 30 mM. (*a*), Rat-liver microsomes, 0·25 ml.; rat-liver supernatant, 0·5 ml.; (*b*), microsomes, 0·25 ml.; D-glucuronolactone, 10 mM; sodium glucuronate, 10 mM; (*c*), supernatant, 0·5 ml. and substrate as in (*b*). Incubation was for 2 hr. at 37° in air.

		Ascorbic acid synthesized (μmole)	
	Concn. of substrate (mM)	D-Glucurono-lactone	Sodium glucuronate
(*a*)	None	0	0
	2	0·05	0·06
	4	0·15	0·16
	10	0·15	0·16
	25	0·10	0·14
	Concn. of supernatant (ml.)		
(*b*)	None	0	0
	0·10	0·02	0·03
	0·25	0·08	0·10
	0·50	0·16	0·16
	1·00	0·17	0·18
	1·50	0·16	0·17
	Concn. of microsomes (ml.)		
(*c*)	None	0	0
	0·10	0·08	0·10
	0·25	0·16	0·15
	0·50	0·23	0·24
	1·00	0·30	0·31
	1·50	0·36	0·37

Table 8. *Relative effects of soluble supernatant, reduced triphosphopyridine nucleotide and potassium cyanide on the conversion of* D-*glucuronolactone and sodium* D-*glucuronate into* L-*ascorbic acid by rat-liver microsomes*

Test system: microsomes and soluble supernatant (rat liver), 0·25 ml. and 0·5 ml. respectively; TPNH, mM; nicotinamide, 30 mM; KCN, 50 mM, where used. Incubation was for 2 hr. Other conditions were as stated in Table 7.

System	Ascorbic acid synthesized (μmole)	
	D-Glucoronolactone	Sodium glucuronate
Microsomes	0	0
Microsomes plus supernatant	0	0
Microsomes plus supernatant plus TPNH	0·16	0·15
Microsomes plus supernatant plus TPNH plus KCN	0·35	0
Microsomes plus supernatant plus KCN	0·36	0
Microsomes plus KCN	0·62	0

Table 9. *Effect of different concentrations of potassium cyanide on the relative conversion of* D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid by microsomes and soluble supernatant from rat liver*

Conditions were as stated in Table 8.

Concn. of KCN (mM)	Ascorbic acid synthesized (μmole)	
	D-Glucurono-lactone	Sodium glucuronate
0	0·16	0·16
1	0·15	0·16
2	0·16	0·15
5	0·17	0
50	0·35	0

tration of the microsomes, however, the synthesis increases to a small extent (Table 7). When 50 mM-KCN is added to a system containing the microsomes, the soluble supernatant and TPNH, the synthesis from D-glucuronolactone is significantly enhanced and the requirement for TPNH no longer exists. Omission of the soluble supernatant further enhances the synthesis. On the other hand cyanide at such a high concentration (50 mM) completely inhibits the synthesis from sodium glucuronate (Table 8). The effects of different concentrations of cyanide on the relative conversion of D-glucuronolactone and sodium glucuronate in the presence of rat-liver microsomes, rat-liver soluble supernatant and TPNH are shown in Table 9. It may be mentioned that the microsomes or the supernatant obtained from a homogenate prepared with an iso-osmotic KCl solution have been found to behave in a similar way to those prepared with an iso-osmotic sucrose solution. Microsomes and the soluble supernatant from goat liver and chick kidney behave similarly to those from rat liver in the conversion of D-glucuronolactone or sodium glucuronate into L-ascorbic acid.

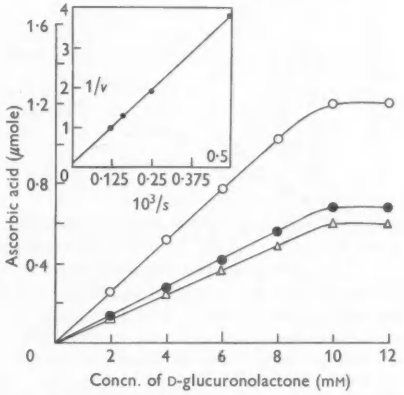


Fig. 4. Effect of varying concentrations of D-glucuronolactone on the synthesis of L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: soluble enzyme, 0·4 ml.; KCN, 50 mM; incubation was for 1 hr. Inset figure represents $1/v$–$1/s$ for D-glucuronolactone with the soluble-enzyme preparation from goat-liver microsomes, where v is μmoles of ascorbic acid formed/hr. and s is D-glucuronolactone in moles/l.

Some properties of the soluble-enzyme preparations obtained from the livers of rat and goat and the kidney of the chick. The effect of variation of the concentration of D-glucuronolactone on the synthesis of L-ascorbic acid by the soluble-enzyme preparations from the goat, rat and chick is shown in Fig. 4. The Michaelis constant for D-glucuronolactone with the goat-liver enzyme, as calculated from the overall reaction, is 93 mM. The Michaelis constants for L-gulonolactone with the soluble-enzyme preparations from the goat, rat and chick, as calculated from Fig. 5, are 10·1 mM, 19 mM and 12 mM respectively. With either D-glucuronolactone or L-gulonolactone as the substrate the

synthesis is proportional to the enzyme concentration (Figs. 6, 7).

In contrast to the great stability of the microsomes, the activity of the soluble-enzyme preparation from goat liver falls sharply on storage at $-15°$ and is completely lost after 6–7 days, and that obtained from the rat liver or the chick kidney is stable only for 1–2 days. As reported for microsomes (Chatterjee *et al.* 1958*c*, 1960*a*), the activity of the soluble-enzyme preparation from goat liver is strongly inhibited by *p*-chloromercuribenzoate, the inhibition being reversed by reduced glutathione. Soluble-enzyme preparations from rat liver and chick kidney behave similarly.

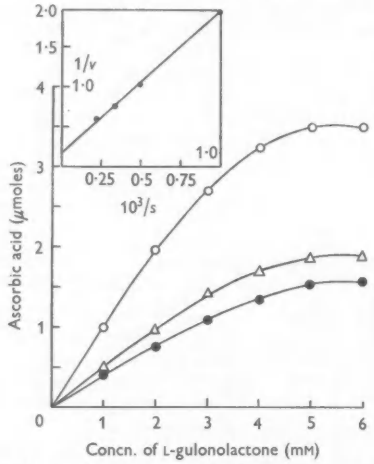


Fig. 5. Effect of varying concentrations of L-gulonolactone on the synthesis of L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: soluble enzyme, 0·4 ml.; incubation was for 1 hr. Other conditions were as stated in the text. Inset figure represents $1/v$–$1/s$ for L-gulonolactone with soluble-enzyme preparation from goat-liver microsomes, where v is μmoles of ascorbic acid formed/hr. and s is L-gulonolactone in moles/l.

Fig. 6. Effect of enzyme concentration on the conversion of D-glucuronolactone into L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: D-glucuronolactone, 10 mM; KCN, 50 mM; incubation was for 1 hr.

Mechanism of oxidation of L-*gulonolactone by the soluble-enzyme preparations from the rat, goat and chick.* During the study of the oxidation of L-gulonolactone by the soluble-enzyme preparations from the rat, goat and chick with the manometric technique, it has been observed that the molar ratio of ascorbic acid formed to oxygen uptake in the presence of added catalase is approximately 2 (Table 10). In the absence of added catalase, this ratio (1·6–1·70) approaches 2 owing apparently to the presence of catalase activity in the enzyme preparation itself. When methanol is added along with the catalase, it will be observed (Table 10) that for 1 mole of ascorbic acid formed 1 mole of oxygen is consumed, with the consequent formation of approximately 1 mole of formaldehyde. The results indicate that in the absence of methanol the hydrogen peroxide formed during the oxidation of L-gulonolactone is decomposed by the catalase and the net oxygen uptake becomes 0·5 mole of oxygen/mole of ascorbic acid formed, as would be expected. In the presence of methanol, however, the hydrogen peroxide is utilized in the oxidation of methanol, producing formaldehyde, and thus the net oxygen uptake becomes 1 mole of oxygen/mole of ascorbic acid formed. With a soluble-enzyme preparation from guinea-pig liver, no oxygen uptake was observed under similar conditions.

Effect of cyanide on the oxidative formation of L-*ascorbic acid by the soluble-enzyme preparation from goat-liver microsomes.* Results showing the effect of cyanide on the oxygen consumption by L-gulonolactone and D-glucuronolactone in the presence of

Fig. 7. Effect of enzyme concentration on the conversion of L-gulonolactone into L-ascorbic acid by soluble-enzyme preparations from goat (○), rat (●) and chick (△). Test system: L-gulonolactone, 5 mM; incubation was for 1 hr.

Table 10. *Oxidation of* L-*gulonolactone by soluble-enzyme preparations from goat, rat and chick*

Methanol and catalase, where used, were 20 mM and 0·015 mg. of haematin equivalent respectively; enzyme preparation was 5 mg. of protein equivalent. Total volume was 2·7 ml. L-Gulonolactone was added from the side arm of the Warburg flask. Incubation was at 37° for 45 min. with an equilibration period of 10 min.

Source of enzyme	Addition	Ascorbic acid formed (μmole) / Uptake of O_2 (μmole)	Formaldehyde (μmole)
Goat liver	L-Gulonolactone	1·70	—
	L-Gulonolactone plus catalase	1·98	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·90
	Catalase plus methanol	—	0·01
Rat liver	L-Gulonolactone	1·60	—
	L-Gulonolactone plus catalase	1·95	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·85
	Catalase plus methanol	—	0·01
Chick kidney	L-Gulonolactone plus catalase	1·85	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·80
	Catalase plus methanol	—	0·01
Guinea-pig liver	L-Gulonolactone plus catalase	0	—
	L-Gulonolactone plus catalase plus methanol	0	0·01
	Catalase plus methanol	—	0·01

Table 11. *Effect of cyanide on the oxygen consumption by* L-*gulonolactone and* D-*glucuronolactone in the presence of a soluble-enzyme preparation from goat liver*

Test system: D-glucuronolactone, 10 mM; L-gulonolactone, 5 mM; enzyme preparation, 0·25 ml.; other conditions were as stated in Table 10.

Substrate	KCN (mM)	Oxygen consumed (μmoles)	Ascorbic acid formed (μmole)
L-Gulonolactone	0	0·98	1·90
L-Gulonolactone	2	1·02	1·85
L-Gulonolactone	50	2·20	0·85
D-Glucuronolactone	0	0·01	0
D-Glucuronolactone	2	0·02	0
D-Glucuronolactone	50	2·04	1·12

Table 12. *Effect of* pH *on the oxidative conversion of* L-*gulonolactone into* L-*ascorbic acid by a soluble-enzyme preparation from goat liver*

Test system: soluble-enzyme preparation, 0·25 ml.; L-gulonolactone, 5 mM; sodium phosphate buffer, 20 mM. Other conditions were as stated in Table 10.

Substrate	pH	Oxygen consumed (μmole)	Ascorbic acid formed (μmole)
L-Gulonolactone	7·0	0·98	1·7
	7·3	0·98	1·6
	7·6	0·95	1·2
	7·9	0·90	0·8

the soluble enzyme preparation from goat-liver microsomes are given in Table 11. Table 11 shows that though a high concentration of cyanide (50 mM) is inhibitory to the conversion of L-gulonolactone into L-ascorbic acid, the oxidation of L-gulonolactone to L-*xylo*hexulonolactone (the identification of which is described in the following section) is not hampered. Similar results have been obtained with microsomes from rat liver and chick kidney. The inhibition in the conversion of L-gulonolactone into L-ascorbic acid has been found to decrease with a decreasing cyanide concentration and no inhibition is observed with 2 mM-KCN. This indicates that the inhibition by cyanide is due probably to the formation of a cyanohydrin derivative of the keto-intermediate, thus arresting its spontaneous conversion into L-ascorbic acid. The amount of oxygen consumed with 50 mM-KCN is about twice that consumed in the absence of cyanide, owing apparently to the inhibition of the catalase activity of the enzyme preparation by cyanide. Table 11 also shows that with D-glucuronolactone there is no oxygen consumption in the absence of cyanide, which indicates that probably no L-gulonolactone is formed under this condition.

Product of oxidation of L-*gulonolactone by the soluble-enzyme preparations from goat, rat and chick.* The immediate product of oxidation of L-gulonolactone by the soluble-enzyme preparations from the livers of the goat and rat and the kidney of the chick has been found to be L-*xylo*hexulonolactone

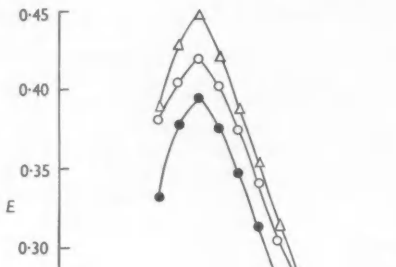


Fig. 8. Absorption-spectra analysis of the *o*-phenylenediamine derivative of L-*xylo*hexulonate. Each cuvette (1 cm.) contains *o*-phenylenediamine derivative of approximately 10–12 μg. of sodium L-*xylo*hexulonate in glass-distilled water. Total volume was 3 ml. △, *o*-Phenylenediamine derivative of an authentic sample of L-*xylo*hexulonate; ●, *o*-phenylenediamine derivative of the synthesized L-*xylo*hexulonate; ○, mixture of the authentic and the synthesized samples.

identified as the *o*-phenylenediamine derivative of L-*xylo*hexulonic acid. L-*xylo*Hexulonolactone but not the corresponding free acid is spontaneously converted into L-ascorbic acid. Hence L-*xylo*hexulonolactone was made to accumulate in the system as sodium L-*xylo*hexulonate by competitive hydrolysis of the lactone keeping the pH of the incubation medium high. At this high pH (7·9) the oxidation of L-gulonolactone was not at all hampered (Table 12), but the relative amount of ascorbic acid formed was small, indicating that the intermediate was trapped in the system. The intermediate thus obtained could, however, be converted into L-ascorbic acid (identified as described in the Methods section) by non-enzymic lactonization in the presence of HCl. The trapped intermediate was converted into the *o*-phenylenediamine derivative, the preparation of which has been described in the Methods section. The *o*-phenylenediamine derivative thus formed was found to be identical with that prepared from an authentic sample of sodium L-*xylo*hexulonate by its typical blue fluorescence in the ultraviolet region and by its paper-chromatographic analysis as described in the Methods section. The identity of the quinoxaline derivative was further confirmed by the measurement of absorption spectra over the region 325–360 mμ. An absorption maximum at 335 mμ with 1·51 as the ratio of the extinctions at 330 and 360 mμ, characteristic of the quinoxaline derivative of an authentic sample of sodium L-*xylo*hexulonate, was obtained (Fig. 8). It may be mentioned that the condensation product of dehydroascorbic acid with *o*-phenylenediamine gives a yellow fluorescence and shows no absorption in the region 325–360 mμ.

DISCUSSION

Site of the enzyme system

The enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid has been found to be located entirely in the microsomal fraction of liver homogenates of the rat and goat and of kidney homogenates of the chick (Table 1). The synthesis by the homogenate is significantly lower than that by the microsomes. This is due to the fact that the soluble supernatant in the homogenate contains the lactonase (as shown in this paper and by Chatterjee *et al.* 1958*c*, 1960*a*), which hydrolyses D-glucuronolactone and L-gulonolactone into the corresponding free acids, the latter being not acted on by the microsomes for the synthesis of L-ascorbic acid.

Effect of cyanide and some other factors on the biosynthesis

Synthesis by rat-liver and chick-kidney microsomes. The conversion of D-glucuronolactone into L-ascorbic acid by the microsomes presumably follows the sequence:

$$\text{D-Glucuronolactone} \xrightarrow{\text{reduction}} \text{L-gulonolactone} \xrightarrow{\text{oxidation}} \text{L-ascorbic acid.}$$

The synthesis from D-glucuronolactone is greatly stimulated in the presence of potassium cyanide in all the species studied. Maximum conversion of D-glucuronolactone into L-ascorbic acid is obtained with a concentration of cyanide of the order of 50 mM, decreasing with a decreasing cyanide concentration in all the species (Fig. 1). With rat-liver and chick-kidney microsomes no appreciable conversion is noted with 5 mM-potassium cyanide. At this concentration of cyanide the synthesis could, however, be stimulated by DPNH, TPNH or reduced GSH (Table 2). DPNH, TPNH and GSH can be replaced more efficiently by a metal-binding agent such as sodium pyrophosphate, αα′-dipyridyl or 8-hydroxyquinoline, or by the boiled supernatant obtained from livers of the rat, goat, guinea pig or the kidney of the chick (Tables 2 and 3). The possibility that cyanide, chelating agent or the

boiled supernatant promotes the synthesis by inhibiting the microsomal lactonase, which hydrolyses D-glucuronolactone to D-glucuronic acid (Chatterjee *et al.* 1958*c* and this paper; Winkelman & Lehninger, 1958; Yamada, 1959), and by thus increasing the availability of the lactone form does not exist since none of these agents has been found in this Laboratory to have any influence on the uronolactonase activity.

Since no cyanide is needed for the conversion of L-gulonolactone into L-ascorbic acid, the function of cyanide lies apparently in the reduction of D-glucuronolactone to L-gulonolactone, which constitutes the first stage of the reaction. The microsomal-enzyme system catalysing the reduction of D-glucuronolactone to L-gulonolactone probably contains some thiol groups, which are activated by cyanide (Chatterjee *et al.* 1958*c*). The rate of reduction of D-glucuronolactone is presumably dependent on the rate of activation of the thiol groups, the latter being dependent on the concentration of the cyanide present in the system, being maximum with 50 mM-potassium cyanide. When, however, the cyanide concentration becomes as low as 5 mM, probably the activation of the thiol enzyme in rat-liver and chick-kidney microsomes is not sufficient to produce L-gulonolactone in the concentration necessary for the production of a detectable amount of L-ascorbic acid. It is likely that the addition of DPNH, TPNH or GSH to such a system stimulates the function of cyanide in promoting the first stage of the reaction, with the consequent formation of an appreciable quantity of L-gulonolactone leading to the synthesis of L-ascorbic acid. However, the formation of L-ascorbic acid in this system can be further enhanced even in the absence of DPNH, TPNH or GSH if any of the chelating agents such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline or the boiled supernatant is added (Tables 2 and 3). Since these agents have been found to promote the conversion of L-gulonolactone into L-ascorbic acid (Chatterjee *et al.* 1960*a*), it would appear that they enhance the overall reaction from D-glucuronolactone by stimulating the second stage of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid. This is supported by the observation in this Laboratory that with a concentration of L-gulonolactone as low as 1 mM, no appreciable synthesis of L-ascorbic acid is brought about by the rat-liver or chick-kidney microsomes; but when any of the aforesaid-chelating agents or the boiled supernatant is added to the system, about 0·3–0·4 μmole of L-ascorbic acid is produced.

DPNH, TPNH or GSH does not further enhance the conversion of D-glucuronolactone into L-ascorbic acid already stimulated by 50 mM-potassium cyanide. This would probably indicate that when the activation of the thiol enzyme is maximum, the effect of the reduced pyridine nucleotide is not realised. Also the addition of any of the chelating agents or the boiled supernatant does not further promote the conversion of D-glucuronolactone into L-ascorbic acid stimulated by 50 mM-potassium cyanide. This is probably due to the fact that cyanide at such a high concentration inhibits the second stage of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid (Chatterjee *et al.* 1960*a*), where the function of the chelating agents lies. The net result is that the stimulating effect of the chelating agent is not produced. This is supported by the observation that in the presence of 50 mM-potassium cyanide any of the aforesaid-chelating agents or the boiled supernatant does not enhance the conversion of L-gulonolactone into L-ascorbic acid by the rat-liver microsomes.

Synthesis by goat-liver microsomes. A low concentration of cyanide (5 mM), by itself, is ineffective with rat-liver and chick-kidney microsomes. But this concentration (5 mM) of cyanide can bring about the conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes and this conversion is not enhanced by the addition of any of the aforesaid agents. Perhaps the thiol groups in the microsomal enzyme from the goat liver are released more readily by a comparatively low concentration of cyanide.

It has recently been found (Chatterjee, Ghosh, Ghosh & Guha, 1959*d*; Chatterjee, Kar, Ghosh & Guha, 1959*e*, 1960*b*) that a microsome-bound lipid cofactor is involved in the oxidation of L-gulonolactone by the rat- and goat-liver microsomes. It has also been observed that goat-liver microsomes are extremely stable and can be stored at $-15°$ for months together without any loss of activity. The microsomes from rat liver and chick kidney, on the other hand, are labile and their activity falls by about 80% on storage at $-15°$ for 5–6 days. The activity, however, can be restored by addition of any of the aforesaid chelating agents. Lipid materials such as α-tocopherol and vitamin K_1 have been found to simulate the accelerating effect of the chelating agents for the conversion of L-gulonolactone into L-ascorbic acid by rat-liver and chick-kidney microsomes. These agents have, however, no influence on the synthesis by goat-liver microsomes apparently because their lipid cofactor is more stable. The action of the chelating agents on the microsomes from rat liver or chick kidney is perhaps to be attributed to the protection of the lipid cofactor against auto-oxidation by these agents. However, the mode of action of these agents and the reason for the difference in behaviour between the liver microsomes from the rat and the goat are not quite clear.

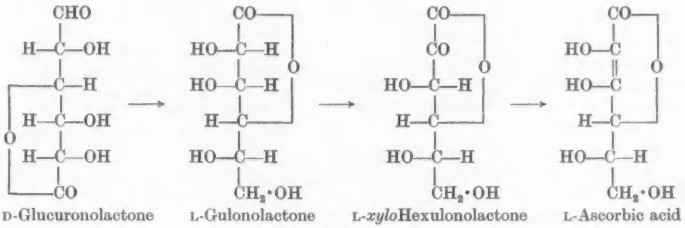
Effect of L-*gulonolactone on the conversion of* D-*glucuronolactone into* L-*ascorbic acid*

It has been reported in this paper that whereas the microsomes do not convert D-glucuronolactone into L-ascorbic acid unless cyanide is present in the incubation media, these do so when L-gulonolactone is added to the system. The amount of ascorbic acid produced is more than that obtained with L-gulonolactone alone and increases with increasing concentration of D-glucuronolactone up to a maximum. Similar results are obtained if increasing concentrations of L-gulonolactone are used with a given concentration of D-glucuronolactone. From Tables 4 and 5 it will be observed that L-gulonolactone in this system behaves similarly to cyanide towards the synthesis of ascorbic acid. It would appear that the cofactor reduced in the oxidation of L-gulonolactone to ascorbic acid functions like cyanide in the enzymic reduction of D-glucuronolactone to L-gulonolactone. The oxidation of L-gulonolactone and the reduction of D-glucuronolactone might appear to be a coupled reaction, but this does not seem strictly to be so, as a catalytic amount of L-gulonolactone in the presence of D-glucuronolactone produces only about double the amount of ascorbic acid that it produces in the absence of D-glucuronolactone and no more. On addition of more L-gulonolactone, however, more ascorbic acid is produced. Besides, this system does not produce ascorbic acid in the complete absence of oxygen. The mechanism of the reaction is not quite clear and is under further investigation.

Relative conversion of D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid*

The microsomal enzyme has a specificity for the lactone forms of the substrates: D-glucuronolactone and L-gulonolactone. The free acids are converted only when the soluble supernatant is present in the system. With sodium L-gulonate the soluble supernatant is needed only to supply the gulonolactonase, which lactonizes L-gulonate into L-gulonolactone, the latter being acted on by the microsomal enzyme. With D-glucuronate, however, a TPN-specific aldehyde dehydrogenase in the supernatant first reduces D-glucuronate to L-gulonate and L-gulonate is then lactonized to L-gulonolactone by the gulonolactonase of the supernatant (Hers, 1956; Hassan & Lehninger, 1956; Ishikawa & Noguchi, 1957; Chatterjee *et al.* 1960*a*). In this paper it has been indicated that an enzyme hydrolysing D-glucuronolactone to D-glucuronate is present in the soluble supernatant. This observation has also been made by Yamada (1959). In the absence of cyanide the amounts of ascorbic acid formed from D-glucuronolactone and from D-glucuronate, in the presence of microsomes, soluble supernatant and TPNH, are almost the same (Tables 7 and 8). This would indicate that D-glucuronolactone is probably first hydrolysed to D-glucuronate and the latter is then acted on by the aldehyde dehydrogenase of the supernatant. Recently a purified TPN-specific aldehyde dehydrogenase, free from the lactonase, has been obtained (Mano, Yamada, Suzuki & Shimazono, 1959) from the soluble supernatant, which reduces D-glucuronolactone to L-gulonolactone. This would indicate also the possibility of direct conversion of D-glucuronolactone into L-gulonolactone, thus eliminating the process of secondary lactonization of the L-gulonate formed from D-glucuronate. Since L-gulonolactone is the direct precursor of L-ascorbic acid, in this case the formation of ascorbic acid would be expected to be higher than that obtained from D-glucuronate. But owing to the presence of a strong gulonolactonase in the supernatant (Chatterjee *et al.* 1960*a*), the L-gulonolactone formed directly from D-glucuronolactone is probably competitively hydrolysed to L-gulonate. Hence, irrespective of the starting material, namely D-glucuronolactone or D-glucuronate, the net amount of ascorbic acid synthesized would represent that formed from the gulonate only. It has been found in this Laboratory that though with a high concentration of L-gulonolactone (5 mM) the synthesis of ascorbic acid in the presence of microsomes and the soluble supernatant is higher than that obtained from gulonate (5 mM) (Chatterjee *et al.* 1960*a*), with a low gulonolactone concentration (1–2 mM) the synthesis from gulonolactone is almost equal to that obtained from the corresponding amount of L-gulonate. This would explain why in the absence of cyanide the amount of ascorbic acid formed either from D-glucuronolactone or D-glucuronate in the presence of microsomes, soluble supernatant and TPNH is almost the same.

The mechanism of reduction of D-glucuronolactone to L-gulonolactone by the microsomal enzyme is apparently different from that of D-glucuronate to L-gulonate by the enzyme in the supernatant. The former is accelerated by the presence of a relatively high concentration of cyanide (50 mM), and no addition of cofactor is required, whereas the latter is completely inhibited at this cyanide concentration and addition of TPNH is necessary. With a low cyanide concentration (1–2 mM; Table 9) this inhibition is not, however, observed. The mechanism of the inhibition by a high concentration of cyanide in this system is not quite clear. It may be that D-glucuronate is converted into L-gulonate via an intermediary formation of D-fructuronate, and that a high concentration of cyanide arrests the reduction of D-fructuronate to L-gulonate by formation of a cyanohydrin derivative of the fructuronate. The



Scheme 1

intermediary formation of D-fructuronate in the conversion of D-glucuronate into L-gulonate in a bacterial system has been reported by Payne & McRorie (1958). Evidence of the formation of an unstable intermediate in the reduction of methyl D-galacturonate to L-galactono-γ-lactone has also been obtained by Mapson & Isherwood (1956). Table 6 shows that the rate of formation of ascorbic acid from D-glucuronate in the presence of soluble supernatant plus microsomes is about one-fourth of that obtained from D-glucuronolactone in the presence of the microsomes alone. The synthesis from the lactone increases with an increasing concentration of the microsomes (Chatterjee *et al.* 1958*c*), whereas that from the free acid does not increase with an increasing concentration of the soluble supernatant; the synthesis from the free acid, of course, appreciably increases with an increasing concentration of the microsomes (Table 7).

Yield of L-*ascorbic acid*

The yield of L-ascorbic acid from D-glucuronolactone is rather low. This is probably due to the fact that the lactone is readily hydrolysed into the free acid by the microsomal uronolactonase (Winkelman & Lehninger, 1958), the free acid being not acted on for ascorbic acid formation. The yield of L-ascorbic acid from L-gulonolactone, which is not hydrolysed by the microsomal lactonase, is considerably higher, up to 35% (Chatterjee *et al.* 1960*a*). With the soluble-enzyme preparation about 50% conversion is obtained (Fig. 7).

Mechanism of oxidation of L-*gulonolactone by the soluble-enzyme preparation*

The oxidation of L-gulonolactone by the soluble-enzyme preparation from the liver microsomes of the rat and goat and the kidney microsomes of the chick has been found to be an aerobic process, molecular oxygen being the ultimate electron acceptor. The products of oxidation have been identified as L-*xylo*hexulonolactone and hydrogen peroxide. Hence the enzymic-reaction pattern involved in the microsomal oxidation of L-gulonolactone can be represented as:

L-Gulonolactone + O_2 → L-*xylo*hexulonolactone + H_2O_2.

Since L-*xylo*hexulonolactone is spontaneously converted into L-ascorbic acid, the microsomal conversion of D-glucuronolactone into L-ascorbic acid in animal tissues may be represented as in Scheme 1. This rules out the possibility, postulated by Grollman & Lehninger (1957), of 3-keto-L-gulonate being the intermediate in the synthesis of ascorbic acid. 3-Keto-L-gulonate has recently been found to be the intermediate in the conversion of L-gulonate into L-xylulose (Ashwell, Kanfer & Burns, 1959).

SUMMARY

1. The enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid in the presence of potassium cyanide (50 mM) has been found to be located entirely in the microsomal fractions of the liver homogenates of the rat and goat and the kidney homogenate of the chick. This conversion requires no added cofactor.

2. A low concentration of cyanide (5 mM), by itself, is ineffective with rat-liver and chick-kidney microsomes for the synthesis of L-ascorbic acid from D-glucuronolactone. But the synthesis is stimulated by the addition of: (*a*) reduced di- or tri-phosphopyridine nucleotide, glutathione, adenosine tri- or di-phosphate, or sodium citrate; (*b*) a chelating agent such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl or 8-hydroxyquinoline; (*c*) the boiled supernatant obtained from the liver tissue of the rat, the goat or the guinea pig or the kidney tissue of the chick. In the absence of cyanide these agents are, however, ineffective. On the other hand, they do not further enhance the synthesis stimulated by 50 mM-potassium cyanide.

3. Potassium cyanide (5 mM) can bring about the conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes. The conversion is not enhanced by the addition of any of the aforesaid agents.

4. The microsomes from the livers of the rat and goat and from the kidney of the chick can convert D-glucuronolactone into L-ascorbic acid even in the absence of cyanide if L-gulonolactone is added.

5. Potassium ferricyanide (mM), but not methylene blue or oxidized glutathione, completely

inhibits the conversion of D-glucuronolactone into L-ascorbic acid by rat-liver microsomes in the presence of 5 mM-potassium cyanide plus sodium pyrophosphate. The conversion of L-gulonolactone into L-ascorbic acid is not inhibited by potassium ferricyanide.

6. The microsomes can act only on D-glucuronolactone and not on the corresponding free acid. The soluble supernatant contains a strong lactonase hydrolysing D-glucuronolactone to D-glucuronic acid and thus inhibiting the cyanide-stimulated microsomal conversion of D-glucuronolactone into L-ascorbic acid.

7. Sodium glucuronate can also be converted into L-ascorbic acid but to effect this synthesis the presence of the soluble supernatant and reduced triphosphopyridine nucleotide along with the microsomes is necessary. This conversion is inhibited by 5 mM or a higher concentration of cyanide, in contrast with the microsomal conversion of D-glucuronolactone into L-ascorbic acid, which is stimulated by cyanide.

8. Soluble-enzyme preparations have been obtained from the microsomal fractions of the liver tissues of the rat and goat and the kidney tissue of the chick. Some properties of these enzyme preparations in the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid have been studied.

9. The oxidation of L-gulonolactone by the soluble-enzyme preparations from the liver microsomes of the rat and goat and the kidney microsomes of the chick is an aerobic process, oxygen being the ultimate electron acceptor. Hydrogen peroxide is formed in the process.

10. The oxidation of L-gulonolactone by microsomes from rat liver and chick kidney and by the soluble-enzyme preparation from goat-liver microsomes is not inhibited even by 50 mM-potassium cyanide.

11. The immediate product of oxidation of L-gulonolactone by the soluble-enzyme preparations obtained from the rat, goat and chick has been identified as L-*xylo*hexulonolactone.

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Effect of Citric Acid-Cycle Intermediates on Oxaloacetate Utilization and Succinate Oxidation

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Evidence that oxaloacetate plays an important rate-regulating role in metabolism through the citric acid cycle is growing, particularly through studies on both animal and plant tissues, demonstrating that the rate of succinate oxidation under different conditions is inversely related to the oxaloacetate concentration and thus influenced by factors affecting the production and removal of the keto acid (Pardee & Potter, 1948; Brummond & Burris, 1953; Tyler, 1955*a*, *b*; Wolff & Ball, 1957; Avron & Biale, 1957; Corwin & Schwarz, 1959). This paper reports the results of examination of some other factors affecting the metabolism of oxaloacetate and its capacity to inhibit succinic dehydrogenase. It will be shown that oxaloacetate utilization is inhibited by the presence of some citric acid-cycle intermediates and this inhibition is reflected in a potentiation of the inhibition of succinate oxidation.

EXPERIMENTAL

Kidney, brain, liver and heart removed from decapitated rats were chilled, rapidly weighed and homogenized (Tyler, 1955*a*) at 0° in 9 parts of 0·9% NaCl containing mM-ethylenediaminetetra-acetic acid (EDTA; disodium salt three times recrystallized). Incubations, in a Dubnoff metabolic shaker, were at 28° (a few were at 38°). The reaction mixture contained 0·5 ml. of the homogenate, 0·2 ml. of 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7·4, 0·1 ml. of 0·25% cytochrome *c*, water to make a final volume of 2 ml. and the substrates and co-factors in the concentrations indicated in the tables. The co-factors were used in excess of the concentrations that gave maximum rates under these conditions, so that they were not rate-limiting. Reaction was stopped at 15 min. by the addition of 2 ml. of ice-cold 10% trichloroacetic acid. 'Total keto acid' was determined colorimetrically (Friedemann & Haugen, 1943), pyruvic acid being used as standard. Utilization rates were calculated from differences in concentrations at zero time and after incubation for 15 min. When α-oxoglutaric acid and oxaloacetate were added together in a reaction mixture the concentrations of each at the end of incubation for 15 min. were determined by a slight modification of the Friedemann & Haugen (1943) procedure, taking advantage of the fact that the 2:4-dinitrophenylhydrazones of oxaloacetate are readily decarboxylated by heating over boiling water to the 2:4-dinitrophenylhydrazones of pyruvate whereas those of α-oxoglutarate are relatively stable to such treatment. After heating, the 'total keto acid' is determined and a portion is extracted with xylol, a solvent which preferentially extracts the 2:4-dinitrophenylhydrazones of pyruvate in a mixture with those of α-oxoglutarate.

Succinoxidase activity was determined manometrically (Tyler, 1955*a*). Cytochrome *c*, adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co. and methods of preparation of material are described elsewhere (Tyler, 1955*a*).

RESULTS

Effect of succinate on oxaloacetate utilization. The addition of succinate (final concentrations 0·01–0·05M) to a reaction mixture containing 3 mM-oxaloacetate caused a significant decrease in the utilization of the keto acid in all tissues studied, the inhibition increasing with increasing concentrations of succinate (Table 1). This inhibition is a true one, not an apparent one due to the formation of new oxaloacetate during succinate oxidation. Under the conditions of these experiments (but with oxaloacetate omitted), 25 mg. of kidney formed 8, 11 and 12 μg. of keto acid from 0·01, 0·02 and 0·05M-succinate respectively. To correct for this new formation, these values were added to the zero-time values. Such corrections are probably an unnecessary precaution; in the presence of added oxaloacetate, succinate oxidation is markedly decreased and so there should be little, if any, new formation of the keto acid. However, the correction is a small one and has little effect on the results.

To examine the nature of this inhibition further a few experiments were carried out with varying concentrations of oxaloacetate, with and without added 0·02M-succinate. Treatment of these results, according to the method of Wilson (1949), by using the expression

$$\frac{V}{V_1} = 1 + \frac{K_s}{K_i}\left[\frac{(I)}{K_s + (S)}\right]$$

gave a plot with a common intercept and slopes suggestive of a competitive type of inhibition (Fig. 1) (K_i 4·7 mM).

Table 1. *Effect of succinate on oxaloacetate utilization*

Beakers contained the following: EDTA (0·25 mM), tris, pH 7·4 (0·02 M), inorganic orthophosphate (1·5 mM), $MgSO_4$ (1 mM), adenosine triphosphate (1 mM), cytochrome *c* (0·05 mM), oxaloacetate (3 mM). The reaction was started by the addition of 0·5 ml. of a 5 % homogenate containing 0·4–0·9 mg. of nitrogen. Oxaloacetate utilization is given as μmoles of the keto acid disappearing in 15 min. at 28° in the presence of 25 mg. (wet wt.) of tissue. Figures in parentheses are percentage changes from control utilization values, without added succinate.

	Concn. of succinate (M)			
Tissue	0	0·01	0·02	0·05
Brain	1·70	1·30 (− 24)	0·95 (− 44)*	0·55 (− 68)*
Liver	2·75	1·20 (− 56)*	0·95 (− 66)*	0·70 (− 73)*
Kidney	3·65	2·40 (− 34)*	1·95 (− 47)*	1·80 (− 51)*
Heart	4·15	2·20 (− 47)*	1·90 (− 54)*	1·50 (− 64)*

* $P < 0{\cdot}01$.

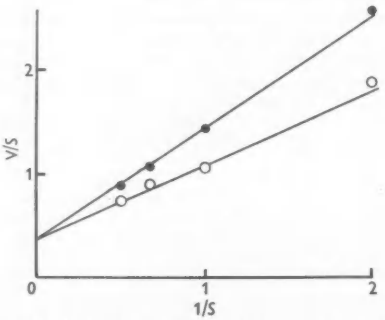

Fig. 1. Inhibition of oxaloacetate utilization by succinate. Kidney homogenate; conditions were as described in Table 1. *S*, Substrate concn. (mM); *V*, rate of disappearance of oxaloacetate (μmoles/15 min.); K_i, 4·7 mM; K_m, 1·86 mM. ○, System without succinate; ●, system with 0·02 M-succinate.

Effect of L-*malate and fumarate on oxaloacetate utilization.* L-Malate and fumarate inhibit oxaloacetate utilization, the former being somewhat more effective than the latter. In both cases the inhibition is greater at 38° than at 28°. Results with L-malate are summarized in Table 2 (the effect of fumarate is similar and is not shown). Corrections were made for possible new formation of oxaloacetate from these substances, as was done for succinate. Increasing the concentration of L-malate increases inhibition but, in contrast with the action of succinate, inhibition is independent of oxaloacetate concentration.

Effect of α-oxoglutarate on oxaloacetate utilization. When these two substances are added together in a reaction mixture the total utilization of keto acid is less than the sum of the individual utilization rates (Table 3). This holds for concentrations of the separate keto acids from 0·5 to 4·0 mM, and the decrease varies from 19 to 37 %. Concentrations of ATP and magnesium are not rate-limiting as the decrease occurs not only in the absence of these cofactors but also when they are present in considerable excess (Table 4). The decrease is largest during the first 5 min. of the reaction and then rapidly falls off (Table 5). The concentration of each keto acid at 15 min. (not shown), found by a modification of the Friedemann & Haugen (1943) method, suggests that an approximately equal percentage decrease in the rate of utilization of both occurs. Also, this inter-relationship between two keto acids is not seen if pyruvate is used in a mixture with oxaloacetate or α-oxoglutarate. In such experiments the resulting utilization of 'total keto acid' is equal to the sum of the individual rates or, under certain conditions, is actually greater.

Effect of citric acid-cycle intermediates on succinate oxidation. Of all the cycle intermediates only oxaloacetate has a striking inhibitory action on succinate oxidation (Table 6). Three intermediates: fumarate, L-malate and α-oxoglutarate, which inhibit the rate of oxaloacetate disappearance, also potentiate the capacity of oxaloacetate to inhibit succinate oxidation (Table 6, lines 7–9).

DISCUSSION

Two points of particular interest are the rate-limiting effect of the presence of other citric acid-cycle intermediates on oxaloacetate utilization and the reflexion of such inhibition on the level of succinate oxidation. Differences in oxaloacetate disappearances in homogenates may be due to enzymes other than the condensing enzyme and all of them may be rate-limiting. It also is possible that a single enzyme may be involved in some of the conditions used here. However, in this report we are chiefly concerned with the effect of various additions on the rate of disappearance of oxaloacetate from the reaction mixture, not with the specific reactions or enzyme system(s) that may be involved. The decrease in utilization occurring in a

Table 2. *Effect of* L-*malate on oxaloacetate utilization*

Beakers contained the reaction mixture as given in Table 1. Oxaloacetate and L-malate concentrations were as indicated. Oxaloacetate utilization is expressed as μmoles of the keto acid disappearing in 15 min. at 38° in the presence of 25 mg. (wet wt.) of kidney. Figures in parentheses are the percentage changes from control utilization without added L-malate.

Concn. of L-malate (mM)	Concn. of oxaloacetate (mM)		
	1	5	10
0	1·45	3·45	5·25
1	1·45	3·30 (−4)	4·40 (−15)
5	1·15 (−23)	2·90 (−17)	3·80 (−28)*
10	0·85 (−41)*	1·95 (−40)*	3·00 (−43)*

* $P < 0{\cdot}01$.

Table 3. *Oxaloacetate and α-oxoglutarate utilization when single and mixed*

Beakers contained the reaction mixture as given in Table 1. Oxaloacetate and α-oxoglutarate concentrations are indicated below. Utilization is given as μg. of keto acid disappearing in 15 min. at 28° in the presence of 25 mg. (wet wt.) of kidney. Line 3 is a theoretical utilization rate obtained by adding the utilization rates in lines 1 and 2.

	Concn. of keto acid (mM)			
	0·5	1·0	2·0	4·0
1. Utilization of oxaloacetate (when present as single substrate)	110	180	290	510
2. Utilization of α-oxoglutarate (when present as single substrate)	70	105	120	125
3. Theoretical utilization (when mixed)	180	295	410	635
4. Actual utilization found when oxaloacetate and α-oxoglutarate are mixed	145	205	280	495
5. $\frac{\text{Line 3} - \text{line 4}}{\text{Line 3}} \times 100$	−19%*	−31%*	−32%*	−22%*

* $P < 0{\cdot}01$.

Table 4. *Effect of adenosine triphosphate and magnesium on the utilization of oxaloacetate and α-oxoglutarate*

Keto acid concentration in the beaker was 2 mM. Other conditions are as given in Table 3. Utilization is given in μg. of keto acid disappearing in 15 min. in the presence of 25 mg. (wet wt.) of kidney.

	Without additional ATP or Mg^{2+} ions	With 5 mM-ATP and 5 mM-Mg^{2+} ions
1. Utilization of oxaloacetate when present as single substrate	225	295
2. Utilization of α-oxoglutarate when present as single substrate	35	95
3. Theoretical utilization (when mixed)	260	390
4. Utilization found when both keto acids are mixed	170	245
5. Percentage change	−35*	−37*

* $P < 0{\cdot}01$.

mixture of α-oxoglutarate and oxaloacetate may be due to a limited availability of one or more cofactors common for the utilization of both keto acids. However, a combination of 0·5 mM-oxaloacetate and 0·5 mM-α-oxoglutarate shows a total utilization rate less than that found for oxaloacetate at double the concentration (1 mM), despite a large excess of all known cofactors. Similar effects are found with other concentrations (Table 3). Thus it would appear that the common rate-limiting factor(s) that may exist for these two keto acids is not one of the cofactors added in this experiment.

Large amounts of succinate can inhibit oxaloacetate utilization. This effect also could be mediated through a variety of secondary reactions, all of which may be rate-limiting. The results (Fig. 1) also suggest the possibility that the two substrates may have an affinity for each other's enzymes or for a common enzyme. However, if such a mutual affinity exists oxaloacetate is a much more powerful inhibitor of succinate oxidation (K_i 1·5 μM; Pardee & Potter, 1948) than succinate is of oxaloacetate (K_i 4·7 mM; Fig. 1).

This study is part of an investigation of the possibility that oxaloacetate is an important rate-regulator of oxidative metabolism through the citric acid cycle. Therefore some relation of these results to this hypothesis is indicated. The idea of a regulatory mechanism at the enzyme–substrate level has been considered by others (Rosenthal, 1937; Pardee & Potter, 1948; Davies & Kun, 1957). Although many reactions may be rate-limiting on oxidative metabolism it has been shown that oxaloacetate has a number of characteristics that allow it to play a major role (Tyler, 1957). The inverse relationship between the level of succinate oxidation and the concentration of oxaloacetate is generally represented as a negative 'feed-back'. Fumarate and L-malate check succinic-dehydrogenase activity not only as precursors of oxaloacetate but also by retarding utilization of the keto acid. In view of the well-known negative 'feed-back' between succinic-dehydrogenase activity and oxaloacetate concentration it is somewhat paradoxical to find that succinate, in turn, inhibits the disappearance of oxaloacetate, thus tending to cancel out the negative 'feed-back'. Nevertheless, these seemingly paradoxical effects are better understood if it is borne in mind that a striking characteristic of the enzyme system *in vitro* which oxidizes succinate is its tendency to operate unrestrained by mechanisms which control the use of succinate in the cell (Tyler, 1954). This results in very high activity in homogenates but is apparently restrained in the intact cell by an efficient braking system. Oxaloacetate supplies this restraint very effectively, and any condition which interferes with its production or utilization affects the level of succinoxidase activity accordingly. Thus the results of these and other studies (Tyler, 1955*a*; Wolff & Ball, 1957; Corwin & Schwarz, 1959) indicate that there are finely adjusted mechanisms in the cell geared to the level of oxaloacetate which, in turn, controls succinate oxidation under a variety of circumstances. They also suggest that most substances affecting succinate oxidation (except for frank poisons) probably do so indirectly by acting on oxaloacetate metabolism.

Table 5. *Utilization of oxaloacetate and α-oxoglutarate at 5 min. intervals*

Conditions were as given in Table 3; concentration of keto acids was 1 mM; utilization is given in μg. of keto acid disappearing in the presence of 25 mg. (wet wt.) of kidney.

Time (min.)	Oxalo-acetate	α-Oxo-glutarate	Both keto acids	Change (%)
5	80	30	45	−59
10	50	30	70	−13
15	45	35	60	−25

SUMMARY

1. The rate of oxaloacetate utilization by tissue homogenates is inhibited, under certain conditions, by the presence of succinate, L-malate, fumarate and α-oxoglutarate.

Table 6. *Effect of citric acid-cycle intermediates on succinate oxidation*

Warburg flasks contained: 0·5 ml. of a 5% kidney homogenate in 0·9% NaCl, phosphate (0·01 M), pH 7·4, cytochrome *c* (0·05 mM), succinate (0·02 M). Other substrates were added as indicated. The final reaction mixture volume was 2 ml.; temperature, 38°; gas phase, air. After a preliminary equilibration period of 10 min. oxygen consumption was measured for 15 min. For the controls (line 1) the oxygen consumption (μl./mg. of N) during the first 15 min. was: liver, 60; kidney, 75; heart, 105. These data are expressed as 100 (line 1) and the results of all experimental conditions are calculated relative to these values.

Additions	Relative consumption of O_2		
	Kidney	Liver	Heart
1. None	100	100	100
2. Oxaloacetate (0·02 mM)	55	50	40
3. α-Oxoglutarate (0·5 mM)	90	85	90
4. Fumarate (0·5 mM)	100	100	100
5. Malate (0·5 mM)	115	90	100
6. Citrate (0·5 mM)	100	105	105
7. Oxaloacetate (0·02 mM) + α-oxoglutarate (0·5 mM)	25*	15*	—
8. Oxaloacetate (0·02 mM) + malate (0·5 mM)	30*	20*	—
9. Oxaloacetate (0·02 mM) + fumarate (0·5 mM)	30*	30*	—

* $P < 0·01$ (difference between line 2 and lines 7–9 respectively).

2. The inhibition by the last-named three substances potentiates the inhibition by oxaloacetate of succinate oxidation.

3. The role of oxaloacetate as a rate-regulator of oxidative metabolism through the citric acid cycle is discussed.

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Stimulation of Fatty Acid Synthesis by Oestradiol *in vitro*

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Previous experiments in this Laboratory demonstrated the presence in human placenta of an enzyme system which responds to oestradiol (Villee & Hagerman, 1953). This system, originally thought to be an *iso*citric dehydrogenase which required diphosphopyridine nucleotide as hydrogen acceptor (Villee, 1955), was subsequently found to require minute amounts of triphosphopyridine nucleotide and was shown to be a transhydrogenase (Talalay & Williams-Ashman, 1958; Villee & Hagerman, 1958). The transhydrogenation reaction, TPNH + DPN → DPNH + TPN, can be coupled to glucose 6-phosphate dehydrogenase as well as to *iso*citric dehydrogenase, and presumably can be linked to any dehydrogenase requiring triphosphopyridine nucleotide (Villee & Hagerman, 1958).

Talalay & Williams-Ashman (1958) suggested that this system is mediated by a 17β-oestradiol dehydrogenase with dual pyridine nucleotide specificity which alternately oxidizes and reduces oestradiol:

$$\text{oestradiol} + \text{DPN} \rightleftharpoons \text{oestrone} + \text{DPNH},$$
$$\text{oestrone} + \text{TPNH} \rightleftharpoons \text{oestradiol} + \text{TPN}.$$

Sum: $$\text{DPN} + \text{TPNH} \rightleftharpoons \text{DPNH} + \text{TPN}.$$

Recent experiments in this Laboratory (Hagerman & Villee, 1959) have shown that the transhydrogenase stimulated by oestrogen and the oestradiol dehydrogenases are separate and distinct enzymes. They differ markedly in their response to thyroxine, to adenosine 2′-monophosphate and to sulphydryl inhibitors; they have different rates of thermal inactivation; and they may be separated physically by electrophoresis on a starch block or on a filter-paper curtain or by adsorption on to and elution from diethylaminoethylcellulose columns. Talalay & Williams-Ashman (1958) had postulated that all steroid hormones produce their effects by way of hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity that rapidly and reversibly oxidize and reduce the steroid. Our recent evidence renders it unlikely that this is the mechanism of action of oestrogens. Other evidence against this theory has been presented by Villee & Spencer (1959) with respect to the mode of action of testosterone and by Bush & Mahesh (1959) with respect to the mode of action of 11β-hydroxysteroids.

The reaction mediated by the transhydrogenase stimulated by oestrogen should be reversible. In experiments reported earlier (Villee & Hagerman, 1958) we were unable to couple the transhydrogenation reaction with a system in which a diphosphopyridine nucleotide-linked dehydrogenase was presented with substrate amounts of triphosphopyridine nucleotide and catalytic amounts of diphosphopyridine nucleotide. However, the trans-

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hydrogenase is inhibited by oxidized triphosphopyridine nucleotide. The present paper reports experiments which demonstrate a coupling of the transhydrogenase stimulated by oestrogen with a fatty acid-synthesizing system which requires reduced triphosphopyridine nucleotide. In this reaction, under the special conditions of the system *in vitro*, hydrogen is transferred from reduced diphosphopyridine nucleotide to triphosphopyridine nucleotide and the synthesis of fatty acids is stimulated. These experiments also indicate that the enzymic synthesis of fatty acids in the placenta occurs by way of the biotin-requiring-enzyme system described by Wakil (1958) and by Brady (1958).

MATERIALS AND METHODS

The diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), adenosine triphosphate (ATP) and coenzyme A (CoA) used in these experiments were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) or from Difco Laboratories (Detroit, Mich., U.S.A.) and were 95% pure.

Preparation of fatty acid-synthesizing enzyme

Human placentae (12–20 weeks gestational age) were obtained at vaginal hysterotomy for therapeutic interruption of pregnancy and were used within 5 min. of their removal from the patient's body. A sample (10–20 g.) of placenta was washed twice in 10 vol. of ice-cold 0·15 M-NaCl. The yolk sac was removed, the placenta was cut into small pieces with scissors and the pieces were homogenized in 4 vol. of 0·25 M-sucrose or 4 vol. of potassium phosphate–potassium bicarbonate buffer, pH 9 (0·1 M-K_2HPO_4–0·07 M-$KHCO_3$). The homogenization was carried out in a glass vessel with a Teflon pestle or in a Waring Blendor for 30 sec. The homogenate was centrifuged at 2000 *g* for 10 min. at 5° to remove cellular debris. The supernatant fluid was centrifuged in a Spinco preparative ultracentrifuge for 60 min. at 60 000 *g*. The resulting supernatant fluid was recovered and fractionated by the addition of solid ammonium sulphate. The protein which precipitated when the solution was made 60% saturated with respect to ammonium sulphate was recovered by centrifuging at 10 000 *g* for 15 min. in the cold. The precipitate was dissolved in 5 ml. of 0·04 M-$KHCO_3$ buffer, pH 8·5, and glutathione was added to a final concentration of 1 mM. This solution was dialysed for 2 hr. in the cold against 1 l. of 0·04 M-$KHCO_3$. The protein solution after dialysis was frozen and stored overnight at −20°. It was then thawed and centrifuged at 1000 *g* for 10 min. The resulting supernatant solution was used in these studies.

Enzymic synthesis of malonyl-coenzyme A

An enzyme system for the activation of malonic acid has been found in acetone-dried preparations of pig heart (Beinert & Stansly, 1953), in rat tissues (Nakada, Wolfe & Wick, 1957) and in human placenta (Hosoya & Kawada, 1958). An acetone-dried powder of pig-heart or human placenta was dissolved in 10 vol. of a solution containing 0·02 M-$NaHCO_3$, 1 mM-sodium ethylenediaminetetraacetate, 1 mM-glutathione and 1 mM-potassium malonate. The fraction precipitating between 40 and 60% saturation with ammonium sulphate was prepared according to the directions of Green, Goldman, Mii & Beinert (1953). This preparation contains a malonate-activating enzyme which requires ATP, CoA, Mg^{2+} ions and malonate. The enzyme preparation was incubated at 37° for 30 min. in a mixture containing 100 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8·2, 10 μmoles of $MgCl_2$, 100 μmoles of potassium malonate, 0·2 μmole of CoA, 10 μmoles of ATP, 10 μmoles of glutathione, 500 μmoles of hydroxylamine and 1·5 mg. of purified pig-heart or placenta enzyme. The product of the reaction was extracted and chromatographed according to the directions of Stadtman & Barker (1950) and Hayaishi (1955). A single hydroxamic acid derivative with R_F 0·33 was detected when the chromatograms were developed with water-saturated butanol. The mobility of this derivative corresponded exactly with that of authentic monomalonyl hydroxamic acid chromatographed simultaneously.

Large-scale incubations were performed as described by Brady (1958), with 10 times the quantity of the materials used in the assay system described above but containing a total of 10 μmoles of CoA. The reaction was terminated by the addition of 2 m-equiv. of acetic acid and the acidified mixture was heated at 100° for 1 min. The resulting suspension was centrifuged and the supernatant solution was recovered and freeze-dried. The dried residue was dissolved in a minimal volume of water and applied to a sheet of Whatman no. 3 filter paper. The chromatogram was developed with a solution of equal volumes of ethanol and 0·1 M-acetic acid–potassium acetate buffer, pH 4·5. After the paper had been dried a small strip was cut out and treated with nitroprusside reagent (dilute ammoniacal solution of sodium nitroprusside) to detect the CoA derivative. The material was eluted with water from the remainder of the chromatogram, and concentrated by freeze-drying and taken up in a small volume of water. A total of 2·2 μmoles of malonyl-CoA was recovered, measured by the method of Lipmann & Tuttle (1945).

RESULTS

Stimulation by oestradiol of the synthesis of long-chain fatty acids

The synthesis of fatty acids by the enzyme system prepared from the placenta was measured by the incorporation of [1-^{14}C]acetate into the lipid fraction. The following components were placed in a small glass-stoppered test tube: coenzyme A, 0·02 μmole; ATP, 2·5 μmoles; Mn^{2+} ion, 0·02 μmole; *iso*citrate, 6·0 μmoles; TPN, 0·2 μmole; DPN, 0·2 μmole; glucose 1-phosphate, 15·0 μmoles; lipoic acid, 0·15 μmole; glutathione, 8·0 μmoles; [1-^{14}C]-acetate, 4 μmoles; purified human placental enzyme, 2·0 mg.; potassium phosphate buffer, pH 6·5, 30 μmoles. The final volume of the incubation mixture was 0·5 ml. To one of each pair of vessels an aqueous ethanol solution of oestradiol was added to a final concentration of 4 μM.

The tubes were flushed gently with nitrogen for 30 sec., stoppered and incubated in a water bath at 38° for 2 hr. The reaction was then stopped by the addition of 0·5 ml. of ethanolic 10% KOH and the tube was placed in a boiling-water bath for 30 min. When saponification was complete, 0·5 ml. of 2N-HCl was added to bring the pH to between 2 and 3. The free fatty acids were extracted by adding 4 ml. of *n*-pentane and shaking the tightly stoppered tube for 15 min. After the two phases had separated, the pentane layer was transferred to a 15 ml. graduated centrifuge tube with a medicine dropper. The extraction was repeated with two additional 4 ml. portions of pentane. The pooled pentane extracts were concentrated to 1 ml. in a stream of warm air. Samples (50 μl.) of the pentane solution were plated on aluminium planchets and dried. The radioactivity on the planchet was then measured in a gas-flow counter. The measured radioactivity represents the amount of acetate incorporated into long-chain fatty acids, since only long-chain fatty acids are extracted into the pentane under these conditions. From the specific activity of the substrate acetate, and the total counts/min. in the long-chain fatty acids isolated at the end of the incubation, it was calculated that, without added oestradiol, 0·42 μ-mole, and in the presence of oestradiol (4 μM) 0·51 μmole, of acetate was incorporated into fatty acid during the incubation for 2 hr.

A sample of the pentane extract was transferred to filter paper and developed with ethylene glycol–diethyl ether–propionic acid–water solvent by the method of Benson *et al.* (1950). The radioactivity was located by prolonged exposure of the chromatogram to X-ray film. The spot had an R_F identical with that of palmitate.

These experiments indicated that oestradiol may accelerate the incorporation of [1-^{14}C]acetate into long-chain fatty acids. The first step in the synthesis of fatty acids is the carboxylation of acetyl-CoA to yield malonyl-CoA, catalysed by a biotin-containing enzyme which requires ATP, Mn^{2+} and HCO_3^- ions. The enzyme fraction (containing 1 mg. of protein) prepared from pre-term human placentae was incubated at 37° in an atmosphere of nitrogen in a system containing 0·5 μmole of acetyl-CoA, 5 μmoles of phosphate buffer, pH 7·4, 5 μmoles of ATP, 5 μmoles of $KH^{14}CO_3$ (10^5 counts/min.), 1 μmole of $MnCl_2$, 0·05 μmole of biotin, with and without added oestradiol (final concn. 4 μM). After incubation for 30 min. 300 μmoles of hydroxamic acid were added and the system was incubated for 10 min. Ethanol (5 vol.) was added, the mixtures were centrifuged and the supernatant fluid was evaporated to dryness. The dry residue was extracted with 2 ml. of acetone, the acetone extract was concentrated under nitrogen to 0·1 ml., and this was applied to paper for chromatography. By the method of Stadtman & Barker (1950) malonyl monohydroxamate was identified by its R_F (0·35). The radioactivity of the malonyl monohydroxamate was the same whether or not oestradiol was added, which indicates that the hormone has no effect on the enzymic synthesis of malonyl-CoA.

The second step in fatty acid synthesis is a condensation of malonyl-CoA with acetaldehyde to yield β-hydroxybutyryl-CoA (Brady, 1958) or of malonyl-CoA with acetyl-CoA (Wakil, 1958) to yield butyryl-CoA. Reaction mixtures were prepared containing 25 μmoles of phosphate buffer, pH 7·4, 5 μmoles of KF, 1 μmole of $MnCl_2$, 5 μmoles of ATP, 0·5 μmole of CoA, 5 μmoles of glutathione, 15 μmoles of substrate and 1 mg. of placental enzyme, in a total volume of 1 ml. The mixtures were incubated for 30 min. at 37°, and then 300 μmoles of hydroxylamine hydrochloride were added to terminate the reaction. The mixture was kept at room temperature for 10 min. and then 5 vol. of ethanol was added; the mixtures were centrifuged and each supernatant solution was evaporated to dryness. The residue was extracted with 2 ml. of acetone, the solution was concentrated to 0·1 ml. under nitrogen, transferred to filter paper and developed with butanol–propionic acid–water solvent (Benson *et al.* 1950). In this system, the R_F values of the hydroxamic acid derivatives of malonic acid, acetic acid, β-hydroxybutyric acid and butyric acid are 0·30, 0·51, 0·74 and 0·81 respectively.

When the incubation was carried out with malonate alone or with malonate plus acetate as substrates, β-hydroxybutyrate was formed, as indicated by the production of a hydroxamic acid derivative with R_F 0·75. With malonate and acetaldehyde as substrate no such derivative was formed. With malonate and acetate as substrate an additional hydroxamic acid derivative, with R_F 0·66, was obtained. This was not identified but is probably some intermediate in the reaction system. With malonate alone as substrate, both acetate and β-hydroxybutyrate were formed. Thus the protein prepared from placenta contains enzymes for the decarboxylation of malonate to form acetate as well as ones for the condensation of malonyl-CoA and acetyl-CoA to yield β-hydroxybutyrate.

The condensation step is followed by a reductive step which requires reduced triphosphopyridine nucleotide (TPNH; Wakil, 1958). Since oestradiol has been shown to stimulate a specific pyridine nucleotide transhydrogenase (Hagerman & Villee, 1959), it seemed possible that the stimulation with oestradiol observed in the synthesis of long-chain fatty acids from [1-^{14}C]acetate resulted from a

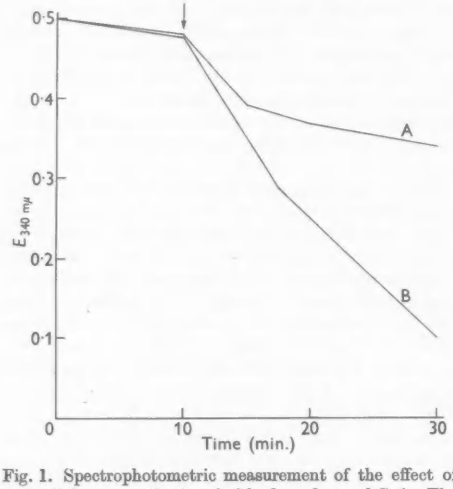

Fig. 1. Spectrophotometric measurement of the effect of oestradiol on synthesis of β-hydroxybutyryl-CoA. The reaction mixture contained 150 μmoles of potassium phosphate buffer or tris buffer (pH 7·0), 0·25 μmole of DPNH, 0·5 μmole of $MnCl_2$, 0·2 μmole of acetyl-CoA and malonyl-CoA. One cuvette (line B) contained oestradiol, final concn. 4 μM. Line A gives extinction of control cuvette to which no oestradiol was added. After incubation for 10 min. (at arrow), 0·01 μmole of TPN was added. Total volume in each cuvette was 3 ml. Temperature was 22°.

stimulation of transhydrogenase coupled with the TPNH-linked reductive step. To test this possibility, a reaction mixture was set up in which fatty acid synthesis was followed spectrophotometrically by the oxidation of reduced diphosphopyridine nucleotide (DPNH) in the presence of the transhydrogenase and catalytic amounts of TPN. Under these conditions the reaction was accelerated by the presence of oestradiol in a final concentration of 4 μM (Fig. 1). This indicates that the pyridine nucleotide transhydrogenase stimulated by oestrogen catalysed the transfer of hydrogen from DPNH to TPN and that the TPNH formed was then reoxidized in the course of the synthesis of β-hydroxybutyrate from acetyl-CoA and malonyl-CoA.

DISCUSSION

The evidence presented here shows that the placenta contains enzymes for the synthesis of fatty acids by the condensation of acetyl-coenzyme A and malonyl-coenzyme A, by reactions similar to those described by Wakil (1958). A partially purified enzyme preparation from human pre-term placentae contains both the enzymes for fatty acid synthesis and the transhydrogenase stimulated by oestrogen. Under conditions in which the supply of TPNH is made rate-limiting but in which there is an adequate supply of DPNH, the synthesis of fatty acids can be stimulated by the addition of oestradiol to the system. The present experiments indicate that this results from a coupling of the TPNH-requiring reductive step which follows the condensation of malonyl-coenzyme A with acetyl-coenzyme A and the pyridine nucleotide transhydrogenase stimulated by oestrogen which in this instance catalyses the transfer of hydrogen from DPNH to TPN. Previous attempts in this Laboratory to demonstrate the transfer of hydrogen in this direction had been unsuccessful (Villee & Hagerman, 1958). However, in the former experiments, substrate amounts of oxidized TPN were supplied to a system containing a DPN-specific dehydrogenase, the transhydrogenase stimulated by oestrogen and catalytic amounts of DPN. Under these conditions the transhydrogenase is inhibited by the oxidized TPN. A TPN-linked glutathione reductase can be coupled with the oestrogen-sensitive transhydrogenase and under these conditions the latter transfers hydrogen from DPNH to TPN (Talalay, Hurlock & Williams-Ashman, 1958). Again, this could be demonstrated only when the concentration of TPN was kept extremely low.

In other experiments (Villee, Hagerman & Joel, 1960) with placenta slices incubated in a medium containing ^{14}C-labelled acetate or glycine, the rate of incorporation of the ^{14}C into fatty acids as well as into proteins and purines was stimulated by the addition of oestradiol. In the intact cell, with most of the TPN in the reduced state and most of the DPN in the oxidized state (Glock & McLean, 1955), it seems unlikely that oestradiol could increase the rate of fatty acid synthesis by increasing the concentration of reduced TPN. Instead, the evidence suggests that the stimulation of the transhydrogenase by oestrogen leads to an increase in the amount of biologically useful energy available to the cell and that this secondarily leads to an increase in the rates of endergonic reactions such as the synthesis of fatty acids, proteins and purines. The present experiments are of interest in showing that under certain conditions *in vitro* the synthesis of fatty acids can be coupled to transhydrogenase which provides the requisite TPNH. They should not be taken as evidence that the oestrogen stimulation of fatty acid synthesis in the intact placental cell necessarily occurs by way of the same mechanism.

SUMMARY

1. Evidence is presented that the human placenta contains enzymes for the carboxylation of acetyl-coenzyme A to yield monomalonyl-coenzyme

A and for the condensation and reduction of these two to yield β-hydroxybutyric acid.

2. These enzymes, which are located in the non-particulate fraction of the cell, were partially purified by ammonium sulphate fractionation.

3. The coupling of the fatty acid-synthesizing system to the transhydrogenase stimulated by oestrogen was demonstrated by the increased rate of conversion of [^{14}C]acetate into labelled fatty acid and by the increased rate of oxidation of reduced pyridine nucleotide when oestradiol was added to the system.

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The Yellow Pigmentation of Bull Semen and its Content of Riboflavin, Niacin, Thiamine and Related Compounds

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Semen is usually white or slightly creamy in colour, but during the course of unrelated studies on the physiology of spermatozoa (White, 1954) the semen from one particular Friesian bull was found to be quite yellow. The colour was confined to the plasma fraction and u.v. light produced a green fluorescence similar to that emitted by riboflavin. By visual examination, the colour of the specimen was comparable with that of an aqueous solution containing at least 10 μg. of riboflavin/ml.

Much lower values for the riboflavin content of bull semen were quoted by VanDemark & Salisbury (1944), who were presumably dealing with unpigmented specimens. The reports of Brochart (1952), Kaemmerer & Krampitz (1955) and the analyses of Mann (1954) suggest that the yellow pigmentation of bull semen may be attributed largely to riboflavin.

Recently it has been possible to obtain several samples of very yellow bull semen, and these have provided material for more extensive investigations into the nature of the pigmentation and its effect on spermatozoa (see White & Lincoln, 1958).

EXPERIMENTAL

Semen and blood. Semen was collected from 13 bulls by the use of an artificial vagina; the semen from five was yellow and the remainder white. Frequently, two ejaculates were obtained from each animal within a few minutes. The samples were protected from light during and after collection, and precautions were taken to prevent any contact with cork, plastic or rubber. The semen was either cooled slowly to 10° or frozen to −79° before transport to the laboratory. Seminal plasma was separated from the spermatozoa by centrifuging. Spermatozoa counts, made with a haemocytometer under high power, were done in duplicate on semen samples diluted with 3% (w/v) NaCl in blood-cell pipettes.

Blood was collected from the jugular vein and the serum that separated from the clot after standing in the dark was used.

Fructose, oxygen uptake and lactic acid. Fructose and the rate of fructolysis in 0·125 M-phosphate buffer, pH 7·4, prepared by dissolving 3·5 g. of Na_2HPO_4 in 190 ml. of water and adding 10 ml. of N-HCl, were estimated by the method of Mann (1948), 20 min. at 80–85° being allowed for colour development (White, 1959).

Oxygen uptake was measured manometrically by the direct Warburg technique, calcium-free Krebs–Ringer phosphate diluent being used (0·132 M-NaCl, 5 mM-KCl, mM-KH_2PO_4, mM-$MgSO_4,7H_2O$, 10 mM-Na_2HPO_4). Spermatozoa were washed twice by diluting about 4 ml. of semen to 10 ml. in a tapering 15 ml. centrifuge tube and centrifuging twice at 1700 rev./min. (about 350 *g*) for 15 min.; the supernatant was removed after each centrifuging and replaced by an equal volume of diluent.

Lactic acid was estimated by the method of Barker & Summerson (1941).

Chromatography and electrophoresis. For chromatography or electrophoresis 1 ml. of seminal plasma was deproteinized by adding 6 vol. of ethanol and centrifuging. The solvent was removed at room temperature in a vacuum desiccator, the residue dissolved in 0·4 ml. of water and the solution clarified by centrifuging in a haematocrit tube. The solution (10 μl.) was applied to Whatman no. 1 chromatography paper along with 10 μl. of the following marker solutions (0·1 μg./μl.); riboflavin, riboflavin phosphate, flavinadenine dinucleotide, lumichrome and lumiflavin. The following solvents were used for the development of the chromatograms: 5% (w/v) Na_2HPO_4 (Carter, 1950); butan-1-ol–acetic acid–water (4:1:5), top phase (Cramer, 1948); phenol–butan-1-ol–water (160:30:100, w/v/v), lower phase (Dimant, Sanadi & Huennekens, 1952); methanol (Yanagita & Foster, 1956). The first three solvents were used in descending systems and the methanol in an ascending one; after drying, the spots were examined under a u.v. lamp. The electrophoresis procedure of Cerletti & Siliprandi (1955) was used.

Absorption spectrum. For the determination of the absorption spectrum of the yellow pigment, 4 ml. of seminal plasma was deproteinized with 30 ml. of ethanol. After evaporation, the residue was dissolved in 0·3 ml. of water and streaked on Whatman no. 1 chromatography paper. The chromatogram was developed with butan-1-ol–acetic acid–water and the yellow strip eluted with water by the technique of Dent, Stephen & Steward (1947). The absorption spectrum was determined in a Uvispek spectrophotometer and compared with that of riboflavin (5 μg./ml.).

Estimation of vitamin B. The quantity of seminal plasma taken for analyses varied from 0·05 to 1·00 ml. depending upon the content of the B vitamins; 5 ml. of blood serum was used.

Thiamine, riboflavin and niacin were estimated by the procedures described in the publication, *Determination of Vitamin B_1, B_2 and Niacin in Foodstuffs* (F. Hoffman-La-Roche and Co. Ltd., Vitamin Department, Basle, 1955).

Thiamine was adsorbed onto Decalso and eluted with acidified potassium chloride before conversion into thiochrome with alkaline ferricyanide (Association of Vitamin Chemists, 1951). Thiamine loss on the surfaces of the laboratory glassware was minimized by observing the precautions described by Farrer (1953).

For the estimation of riboflavin and chloroform-soluble flavin, samples of plasma were acidified with 0·2 N-H_2SO_4, treated with 3% (w/v) $KMnO_4$ and decolorized with 3% (w/v) H_2O_2. After extraction with $CHCl_3$, the riboflavin in the aqueous layer was adsorbed onto kieselguhr, eluted with 2% (w/v) NaOH and converted into lumiflavin by irradiation with u.v. light from a Phillips 160 w bulb, type 57270 E/21. The riboflavin content of the sample was then estimated fluorometrically by comparison with a known amount of pure riboflavin treated similarly. A portion of the $CHCl_3$ layer was transferred to a cuvette for the estimation of the chloroform-soluble fluorescent material.

The Lumetron (model 402 EF) fluorimeter was used and 1 μg. of pure riboflavin or thiamine provided a suitable comparison standard. The instrument was modified by the appropriate attachments for the colorimetric determination of niacin. Niacin was estimated by the reaction with cyanogen bromide using 50 μg. of pure niacin as a standard.

Statistical analysis. Where necessary the statistical significance of the results have been assessed by the *t* test or analysis of variance (*F* test). In the latter case, sums of squares attributable to differences between ejaculates and treatments have been isolated and the interaction mean square used as the error term.

RESULTS

Preliminary observations

The production of yellow semen seems characteristic of the bulls studied, since many samples collected over several years have been pigmented. The animals were located in widely scattered parts of Australia, New Zealand and the U.S.A., and the semen of other bulls on these farms was quite normal in appearance. All bulls producing yellow semen were of the Friesian breed and nine Australian and New Zealand bulls were related, as shown in Fig. 1.

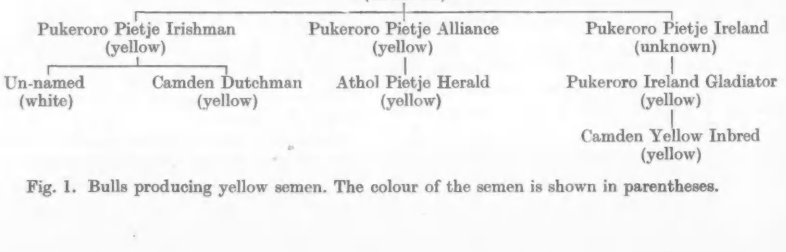

Fig. 1. Bulls producing yellow semen. The colour of the semen is shown in parentheses.

Table 1. *R_F values of bull-semen pigments and markers*

	Solvents			
	Butanol–acetic acid–water	5% Na_2HPO_4	Methanol	Phenol–butanol–water
Seminal plasma extract:				
Yellow spot	0·26	0·27	0·16	0·78
Pale-blue spot	0·13, 0·37, 0·65	0·06	—	0·14
Violet spot	0·86	0·00	—	0·78
Riboflavin (yellow)	0·25	0·30	0·16	0·80
Riboflavin phosphate (yellow)	0·04	0·56	—	0·15
Lumichrome (pale blue)	0·65	0·05	0·28	—
Flavinadenine dinucleotide (yellow)	0·03	0·41	0·00	0·17
Lumiflavin (yellow)	0·41	0·15	—	—

Another Australian bull of a different line (Riverdale King Romeo), producing yellow semen, is known to have two ancestors (Colony Ormsby Sir Romeo 781424 and Colony Vale Sir Romeo Fourth 832252) giving yellow semen, in the U.S.A., and an offspring of the latter (U.W. Grebegga Sir Wayne) also gives yellow semen.

The pigmentation of the semen does not affect the quality of the spermatozoa since all bulls were in use at artificial-insemination centres and were of high fertility. The morphology, fructolysis index and concentration of the spermatozoa were all normal, thus the mean (±S.D.) rate of the fructose breakdown for nine yellow ejaculates was 1·7 ± 0·8 mg./10^9 sperm/hr. and the mean (±S.D.) spermatozoa count of 13 ejaculates was

$$956 \pm 340 \times 10^6/\text{ml.}$$

The pH of the samples ranged from 6·5 to 7·0.

The yellow colour was associated with the seminal plasma rather than the spermatozoa and qualitative tests showed that the pigment had the following properties, which are characteristic of riboflavin (Sebrell & Harris, 1954): (1) a greenish fluorescence when exposed to u.v. light; (2) insoluble in chloroform, ether or benzene; (3) adsorbed on to kieselguhr from acid solutions and eluted with 2% (w/v) NaOH; (4) the colour and fluorescence were destroyed by 3% (w/v) $KMnO_4$ in 2% (w/v) NaOH but not by treatment with 3% (w/v) $KMnO_4$ for 1 min. in acid solution; (5) $Na_2S_2O_5$ destroyed the colour and fluorescence; (6) conc. H_2SO_4 produced a red colour which became yellow on dilution.

On chromatographing, the yellow pigment behaved in a similar manner to riboflavin markers in each of the four solvent systems used (Table 1). Light-blue spots, one corresponding to lumichrome, were also present and also an unknown violet spot which ran well ahead of riboflavin in butan-1-ol–acetic acid–water but remained at the origin in 5% (w/v) Na_2HPO_4.

The absorption spectrum of the yellow pigment is shown in Fig. 2; peaks occurred at 450, 375, 255 and 210 mμ.

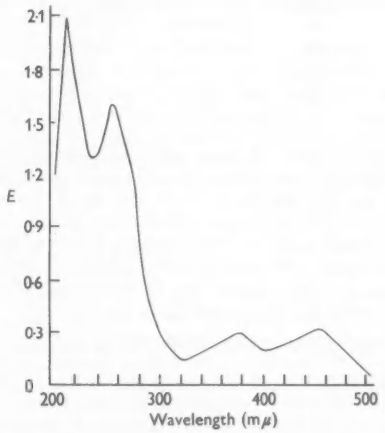

Fig. 2. Absorption spectrum of the yellow pigment in bull seminal plasma.

Quantitative estimations

The riboflavin content of 16 yellow ejaculates from five Friesian bulls was compared with that of nine white ejaculates from nine bulls of other breeds. In the estimation of riboflavin, the preliminary chloroform extract of the yellow semen also fluoresced. This was measured quantitatively and is referred to as chloroform-soluble flavin. It is expressed as the amount of riboflavin which, on irradiation in alkaline solutions, gave an equivalent fluorescence. Thiamine, niacin and fructose analyses were also undertaken to ascertain whether these seminal constituents also differed between the two groups. The results of the vitamin analyses are set out in Table 2. The riboflavin and chloroform-soluble flavin content of the yellow samples were each, on the average, about 40 times that of the colourless semen and sufficient to account for the pigmentation. Both first and second yellow ejaculates obtained at any one time from individual bulls had a high flavin content and the values

Table 2. *Comparison of some constituents of yellow and white bull seminal plasma*

Mean values (μg./ml.) and the population standard deviation are given with the numbers of observations in parentheses.

	Riboflavin	Chloroform-soluble flavin	Niacin	Thiamine
Yellow semen	18·9±6·0 (15)	19·1±6·3 (15)	3·2±1·7 (5)	2·8±1·1 (13)
White semen	0·5±0·2 (8)	0·5±0·6 (8)	1·0±0·2 (4)	1·2±0·8 (9)

Table 3. *Effect of light on the riboflavin and chloroform-soluble flavin content of bull semen*

Values are expressed as μg./ml. before and after incubation of the semen for 3 hr. In Expt. 2 an addition of 20 μg. of riboflavin/ml. of semen was made. C-S flavin, chloroform-soluble flavin.

Expt. no.	Zero time		3 hr. in dark		3 hr. in light	
	Riboflavin	C-S flavin	Riboflavin	C-S flavin	Riboflavin	C-S flavin
1	21·0	19·2	21·9	19·2	0·0	11·2
1	15·5	10·4	12·9	13·2	0·3	12·0
2	40·5	17·0	41·5	18·4	9·7	7·5
2	41·0	16·0	45·5	15·6	7·5	7·5

showed little variation in individual bulls although the samples were collected over a period of several months. The ratio of riboflavin to chloroform-soluble flavin remained fairly constant at unity. Neither the niacin nor thiamine values differed significantly between the groups (Table 2) and the mean fructose value for 14 yellow ejaculates (458 ± 86 mg./100 ml.) was quite normal.

Nature of chloroform-soluble fluorescent material

The exact nature of the chloroform-soluble fluorescent material is at present unknown. Although the chloroform extracts of semen fluoresced strongly under u.v. light in a similar manner to lumiflavin, attempts to demonstrate this substance, by chromatography and electrophoresis, in ethanol and chloroform extracts of semen were not convincing. Baddiley, Buchanan & Carss (1957) have suggested that anhydroriboflavin might occur in tissues but this substance, prepared by their method, could be readily separated from riboflavin by chromatography and is therefore unlikely to be the flavin in question.

The unidentified chromatogram spots (i.e. spots other than the one corresponding to riboflavin) were extracted with hot 0·2N-sulphuric acid and tested in the fluorimeter. Negative readings were obtained for all these spots, so they do not correspond with the unknown chloroform-soluble material.

Stability of semen pigment

When the semen was left on the bench for several hours, the colour faded and experiments were undertaken to ascertain whether this was due to the metabolic activity of the spermatozoa or the action of light. Samples of two freshly collected samples were exposed to direct sunlight at room temperature for 3 hr. and control samples were kept in the dark under similar conditions (Expt. 1, Table 3). The initial and final flavin content was determined. The experiment was repeated with 20 μg. of riboflavin added to each millilitre of semen to check if an equilibrium existed between it and the chloroform-soluble flavin (Expt. 2, Table 3).

There was no breakdown of riboflavin or chloroform-soluble flavin in the dark and no evidence for an equilibrium between the two flavins in the semen. On the other hand, in the presence of sunlight much of the endogenous and added riboflavin was destroyed (F 33·4; $P < 0·01$) and the concentration of chloroform-soluble flavin also decreased (F 9·7; $P < 0·05$). Chromatography of the yellow semen before and after exposure to sunlight revealed a considerable increase in the size of the lumichrome spots.

The photolysis of riboflavin might be expected to yield ribitol. Tests with four bull ejaculates showed that the addition of 1 mg. of ribitol to 1 ml. of washed cells failed to increase the oxygen uptake

Table 4. *Effect of sorbitol and ribitol on the oxygen uptake of bull spermatozoa*

Each manometer flask contained 1 ml. of washed spermatozoa suspension (3·0–6·0 × 10^8 cells) in calcium-free Krebs-Ringer phosphate, incubated in air at 37° for 3 hr. The final concentration of each substrate was 1 mg./ml.

Ejaculate no.	Uptake of O_2 (μl./10^8 spermatozoa)		
	Control	Sorbitol	Ribitol
1	13·4	21·9	14·0
2	27·4	42·3	26·3
3	13·6	24·8	16·5
4	12·7	18·7	13·3
5	19·0	24·6	18·1
Mean	17·2	26·5	17·6

Table 5. *Effect of riboflavin on the metabolism and motility of bull spermatozoa*

Mean values and the population standard deviations are given with the numbers of observations in parentheses. Each vessel contained 1 ml. of spermatozoa suspension (0·9–6·0 × 10^8 cells) in calcium-free Krebs–Ringer phosphate, incubated in air at 37° for 2–3 hr. Motility was scored by microscopic examination by the system of Emmens (1947).

	Uptake of O_2 (μl./10^8 spermatozoa)	Fructose breakdown (μg./10^8 spermatozoa)	Lactic acid production (μg./10^8 spermatozoa)	Motility score
Control	19·7±9·8 (19)	132·1±89 (19)	103±43 (19)	24·2±10·6 (15)
Riboflavin (20 mg./ml.)	10·3±6·1 (19)	104·0±92 (19)	92±53 (19)	17·0±10·8 (15)

(Table 4) under conditions where sorbitol was effective (F 36·5; $P < 0·01$). It seems unlikely therefore that bull spermatozoa are able to oxidize ribitol.

Concentrations of blood riboflavin

Determinations were made of the concentration of riboflavin and of chloroform-soluble fluorescent material in the blood of three bulls which produced yellow semen. The average values were 0·1 and 0·6 μg./ml. respectively.

Effect of riboflavin on the metabolism and motility of spermatozoa

When one part of normal white bull semen was mixed with 1–4 parts of calcium-free Krebs–Ringer phosphate containing fructose at a concentration of 100 mg./100 ml. and sufficient riboflavin to give a final concentration of 20 μg./ml. the oxygen uptake of the suspension was significantly decreased (Table 5; t 3·6; $P < 0·01$).

The mean values for fructose breakdown, lactic acid production and motility are also less in the presence of riboflavin but the response was variable and the differences between control and riboflavin-treated samples are not statistically significant.

DISCUSSION

The results clearly establish that the yellow coloration of some bull ejaculates is due to high concentrations of riboflavin. The pigmentation of the semen is not due to dietary factors, as other bulls under the same conditions of husbandry produce normal semen and the concentrations of blood riboflavin were not elevated. On the other hand, the facts that all the bulls producing yellow semen were of the same breed and many were related, strongly suggest that the characteristic is hereditary and that it may behave like a Mendelian dominant. In view of the blood analyses, the tissues generally of these bulls are probably not capable of synthesizing the flavins. It seems more likely that we may be dealing with a mutation in which the accessory organs, probably the seminal vesicles, have the specific ability to make or concentrate both riboflavin and chloroform-soluble flavin.

Chloroform-soluble lumiflavin is known to be formed from riboflavin when irradiated under alkaline conditions. The chloroform-soluble flavin found in the seminal plasma could not, however, be produced by the photolysis of riboflavin after ejaculation since similar amounts of the two flavins were present even when the most rigorous precautions were taken to exclude sunlight from the semen during collection and handling. Further, bull semen is slightly acid, which would tend to favour photolysis to lumichrome, a process which accompanies the fading of these ejaculates. The enzymic formation of chloroform-soluble flavin from riboflavin after ejaculation is improbable but the accessory organs may contain an enzyme system capable of interconverting riboflavin and the chloroform-soluble material.

The mechanism of the repression of the oxygen uptake of bull spermatozoa by riboflavin is a subject for future investigation. Preliminary tests have indicated that the effect may be largely abolished by the addition of catalase to the diluent. It is possible therefore that the riboflavin may be reduced by the spermatozoa or some seminal constituent and reoxidized on shaking in the Warburg flask with the production of traces of hydrogen peroxide. The oxygen uptake of bull spermatozoa is known to be particularly sensitive to hydrogen peroxide (Tosic & Walton, 1950; Wales, White & Lamond, 1959).

The inability of intact bull spermatozoa to utilize ribitol and at the same time to utilize sorbitol (White, 1957) is surprising since sorbitol-dehydrogenase preparations from bull spermatozoa apparently have at least some activity with ribitol as substrate (King & Mann, 1958).

SUMMARY

1. The seminal plasma from a number of otherwise normal Friesian bulls was quite yellow and the evidence suggests the characteristic is hereditary and may behave like a Mendelian dominant.

2. The yellow pigment has been identified as riboflavin which occurred at an average concentration of 19 μg./ml. in pigmented samples compared with 0·5 μg./ml. in colourless samples. Similar amounts of chloroform-soluble fluorescent material were also present.

3. The niacin, thiamine and fructose levels of the yellow samples were not significantly elevated.

4. Exposure to light caused the yellow semen to fade and this was accompanied by the breakdown of riboflavin to lumichrome. Ribitol, which might be expected to form in the process, is not oxidized by bull spermatozoa.

5. When riboflavin was added to normal white ejaculates at a concentration of 20 μg./ml. it decreased the oxygen uptake and decreased the motility of the spermatozoa.

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Metabolism of Amines

2. THE OXIDATION OF NATURAL POLYAMINES BY *MYCOBACTERIUM SMEGMATIS**

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The degradation of natural polyamines by various bacteria has been described by Razin, Gery & Bachrach (1959). *Pseudomonas aeruginosa* was found to oxidize spermine and spermidine, γ-aminobutyric acid and β-alanine being intermediates in the reaction. Roulet & Zeller (1945) had already reported that *Mycobacterium smegmatis* was capable of oxidizing spermine. It was therefore decided to study polyamine oxidation by this organism, and to compare the oxidation pathway with that of *P. aeruginosa* (Razin *et al.* 1959).

The degradations of γ-aminobutyric acid and β-alanine, which are intermediates in the oxidation of the polyamines, are also described in this paper.

* Part 1: Razin, Gery & Bachrach (1959).

MATERIALS AND METHODS

Organisms. Mycobacterium smegmatis strain 10143 was obtained from the American Type Culture Collection. *Pseudomonas aeruginosa* was the strain used previously (Razin *et al.* 1959).

Media. M. smegmatis was grown on the medium of Youmans & Karlson (1947), distributed in quantities of 100 ml. in Roux bottles. For adaptation, spermine was added to the medium to give a final concentration of 0·1%. *P. aeruginosa* was grown on the adaptation medium, as described by Razin *et al.* (1959).

Cell suspensions. M. smegmatis was grown at 37° for 3–4 days. During this incubation period a pellicle was formed on the surface of the medium. This pellicle was dispersed by shaking with glass beads and the resulting cells were washed three times with 0·85% NaCl solution and suspended in 0·067 M-phosphate buffer, pH 7·2; this suspension contained an equivalent of 10 mg. dry wt. of cells/ml. Cell suspensions of *P. aeruginosa* were prepared as described by Razin *et al.* (1959). Dried cell preparations were obtained by drying the cells over P_2O_5 at a pressure of 0·3 mm. Hg in a centrifugal freeze-drier (W. Edwards and Co. Ltd., Crawley, Sussex, England).

Chemicals. Succinic semialdehyde was prepared according to Prescott & Waelsch (1946). Malonic semialdehyde was prepared by the same method, aspartic acid being used instead of glutamic acid. All other chemicals were commercial products.

Manometric methods. Uptake of O_2 was measured by the conventional Warburg manometric technique, with air as the gas phase and a bath temperature of 30° (Umbreit, Burris & Stauffer, 1957). In anaerobic experiments H_2 served as the gas phase.

Identification of amines. Paper chromatography was used for qualitative and quantitative determination (Giri, Radhakrishnan & Vaidyanathan, 1952) of the amines and their degradation products. The cells of the tested reaction mixture were removed by centrifuging and 60 $mm.^3$ of the supernatant was applied to each spot on Whatman no. 1 filter paper. Butanol–acetic acid–water (50:25:25, by vol.; Baker, Harborne & Ollis, 1952) was the standard solvent used. Other solvents included: 70% (v/v) ethanol (Miller & Rockland, 1952); pyridine–acetic acid–water (50:35:15, by vol.; Decker & Riffart, 1950); 95% ethanol–amyl alcohol–water (70:50:30, by vol.). The papers were sprayed with 0·2% (w/v) ninhydrin in butanol. β-Alanine was also identified by the bioassay method of Billen & Lichstein (1949) with *Saccharomyces fragilis* as test organism. Propane-1:3-diamine was separated by passage through an Amberlite IRC 50 column (Bergström & Hansson, 1951).

Identification of succinic and malonic semialdehydes. 2:4-Dinitrophenylhydrazone derivatives of the semialdehydes were prepared and analysed by paper chromatography according to Block, Durrum & Zweig (1958). The solvents used were: butanol–ethanol–0·5 N-ammonia solution (70:10:20, by vol.; El Hawary & Thompson, 1953); butanol–ethanol (80:20, v/v; Cavallini, Frontali & Toschi, 1949); butanol–N-$NaHCO_3$ (66:33, v/v; Seligson & Shapiro, 1952). The papers were sprayed with 2% (w/v) KOH in ethanol.

The semialdehydes were oxidized to the corresponding acids by alkaline H_2O_2 according to Shriner & Fuson (1948).

Identification of succinic acid and malonic acid. The acids were separated and identified by paper chromatography with ethanol–water–ammonia (80:10:10, by vol.) as solvent. The papers were sprayed with 0·04% (w/v) of bromocresol green in ethanol. Hydroxamate derivatives of the organic acids were prepared according to Feigl (1954) and separated by paper chromatography with water saturated with phenol as solvent (Fink & Fink, 1949). The papers were sprayed with 1% (w/v) $FeCl_3$ solution. The acids were also separated and identified by ion-exchange chromatography by the method of Bush, Hulbert & Potter (1952), with Amberlite IRA 400 instead of Dowex 1.

RESULTS

Oxidation of polyamines by washed cells

Cells grown on Youmans–Karlson medium oxidized spermine, spermidine and putrescine after a lag period. The addition of spermine to this medium enhanced the initial rate of oxidation (Fig. 1). Cells grown on spermine were simultaneously adapted to spermidine and putrescine. During the oxidation of spermine 6 μmoles of oxygen were taken up and 2 μmoles of ammonia were set free for each μmole of substrate oxidized. When spermidine served as substrate 8 μmoles of oxygen were taken up and 2·5 μmoles of ammonia released for each μmole of substrate oxidized.

Oxidation of polyamines by freeze-dried cells

No ninhydrin-positive intermediates could be detected by paper chromatography during the oxidation of the polyamines by washed cells. When freeze-dried cells were employed, several ninhydrin-positive compounds accumulated during spermine oxidation. Fig. 2 shows that after 90 min. of incubation all spermine was oxidized, and that compounds corresponding to spots with R_F values 0·13, 0·24, 0·50 and 0·58 were formed, by using the standard butanol–acetic acid–water solvent system.

The compound with R_F 0·13 was identified as spermidine, by using all the solvents employed for the identification of the amines. The compound giving a spot with R_F 0·24 was isolated from the reaction mixture by Amberlite IRC 50 and identified as propane-1:3-diamine by paper chromatography with the standard solvent. The chloroplatinate derivative prepared from this compound (Wrede, 1926) blackened at 230° and sintered at 240° in the same way as the chloroplatinate of propane-1:3-diamine.

The compound having R_F 0·50 was identified as β-alanine by paper chromatography and by bioassay, and that giving the spot with R_F 0·58 was identified as γ-aminobutyric acid by paper chromatography, by using all the solvents employed for the identification of the amines.

Degradation of γ-aminobutyric acid and β-alanine

The fate of γ-aminobutyric acid and β-alanine, which were found to be intermediates in the oxidation of the polyamines by both *P. aeruginosa* (Razin *et al.* 1959) and *M. smegmatis*, was further investi-

gated. Transamination was found to be the first step in their degradation. A suspension of *P. aeruginosa* cells, containing the equivalent of approx. 5 mg. dry wt. of cells, was incubated with 10 μmoles of γ-aminobutyric acid and 10 μmoles of α-oxoglutamic acid in phosphate buffer, pH 7·2, under anaerobic conditions. After 90 min. of incubation, 3·4 μmoles of glutamate were formed, as determined by quantitative paper chromatography. During the transamination reaction succinic semialdehyde was produced. β-Alanine was converted into malonic semialdehyde in the presence of α-oxoglutarate or pyruvate under anaerobic conditions, by washed or freeze-dried cells of *P. aeruginosa*. Under aerobic conditions these semialdehydes were further metabolized. The semialdehydes were identified by paper chromatography of the 2:4-dinitrophenylhydrazone derivatives.

Succinate and malonate were obtained from the corresponding semialdehydes after oxidation with alkaline hydrogen peroxide. The acids formed were separated on an Amberlite IRA 400 column and finally identified by paper chromatography.

Transamination was also shown to be the first step in the degradation of γ-aminobutyric acid and β-alanine by freeze-dried cells of *M. smegmatis*. Freeze-dried cells (50 mg.) were incubated with 20 μmoles of γ-aminobutyric acid and 20 μmoles of α-oxoglutarate in phosphate buffer, pH 7·2, under anaerobic conditions. After 90 min. of incubation, 2·2 μmoles of glutamate were produced as determined by quantitative paper chromatography. During the transamination reaction succinic semialdehyde was produced. When β-alanine was used instead of γ-aminobutyric acid, malonic semialdehyde was formed. Under aerobic conditions succinate and malonate were formed from γ-aminobutyric acid and β-alanine respectively. The acids formed were separated from the reaction mixture by Amberlite IRA 400, and finally identified as their hydroxamate derivatives.

Effect of inhibitors on polyamine oxidation

The effect of various inhibitors on the oxidation of spermine by *M. smegmatis* is shown in Table 1. Semicarbazide and *iso*nicotinic acid hydrazide inhibited the oxidation of spermine. Dihydrostreptomycin (0·04 mM) did not inhibit the oxidation of putrescine. A partial inhibition was obtained with 2·3 mM-dihydrostreptomycin. The growth of the

Fig. 1

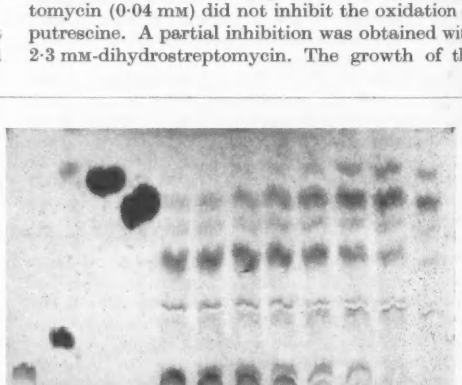

Fig. 2

Fig. 1. Oxidation of polyamines by washed cells of *Mycobacterium smegmatis*. Each Warburg vessel contained 2·5 μ-moles of spermine (○) or spermidine (●), 1 ml. of cell suspension (approx. 10 mg. dry wt.) and 1 ml. of 0·067 M-phosphate buffer, pH 7·2. In the centre well there was 0·2 ml. of 15% (w/v) KOH; the total volume was 3·2 ml. Incubation was carried out in air at 30°. ——, Cells grown on Youmans–Karlson medium; - - - - -, cells grown on Youmans–Karlson medium containing 0·1% (w/v) of spermine.

Fig. 2. Oxidation of spermine by freeze-dried cells of *Mycobacterium smegmatis*. Cells were grown on Youmans–Karlson medium containing 0·1% (w/v) of spermine. Experimental conditions were as given in Fig. 1, but freeze-dried cells (approx. 20 mg. dry wt.) and 2·5 μmoles of spermine were used. The figure represents a photograph of a chromatogram obtained with the butanol–acetic acid–water solvent.

Table 1. *Effect of inhibitors on the oxidation of amines by* Mycobacterium smegmatis

Experimental conditions were as given in Fig. 1, but the cells were grown on Youmans–Karlson medium containing 0·1% (w/v) of spermine and pre-incubated with the inhibitor for 30 min. Experiments were carried out for 3 hr.

Substrate (μmoles)	Inhibitor	Concn. (mM)	Inhibition of O_2 uptake (%)
Spermine 2·5	Semicarbazide	15	86
		7·5	77
	*iso*Nicotinic acid hydrazide	7·5	64
		3·75	8·5
	Dihydrostreptomycin	2·3	60
		0·26	13
		0·13	0
Putrescine 10	Dihydrostreptomycin	2·3	16
		0·04	0

strain used was found to be inhibited by 0·3 μg. of dihydrostreptomycin/ml. in Nutrient Broth (Difco).

DISCUSSION

The degradation of polyamines in *M. smegmatis* is similar to that found in *P. aeruginosa* (Razin *et al.* 1959). γ-Aminobutyric acid, β-alanine and propane-1:3-diamine appear to be intermediates in the reaction. These findings support the view that the pathway described for the bacterial degradation of the polyamines is a general one. Formation of propane-1:3-diamine from spermine by *Haemophilus parainfluenzae* and *Neisseria perflava* has been reported by Weaver & Herbst (1958*a*, *b*).

Transamination was found to be the first step in the breakdown of γ-aminobutyric acid and β-alanine by both *M. smegmatis* and *P. aeruginosa*. The transamination of γ-aminobutyric acid by *Mycobacterium avium* has already been described (Shoji, Mori & Ito, 1958). Succinic and malonic semialdehydes obtained as transamination products were further oxidized by freeze-dried cells of *M. smegmatis* to succinate and malonate respectively. These acids were not detected when freeze-dried cells of *P. aeruginosa* were used (U. Bachrach, unpublished work). Oxygen uptake during the oxidation of the polyamines by *M. smegmatis* was lower than by *P. aeruginosa*, indicating a difference in the final steps of the degradation of spermine and spermidine by these organisms.

The higher oxygen uptake during the oxidation of spermidine as compared with spermine, already described for *P. aeruginosa* (Razin *et al.* 1959), was found to occur also with *M. smegmatis*. No explanation can yet be given for this observation; similar unexplained high oxygen uptakes during the oxidation of several amines have been reported (Gale, 1942).

The oxidation of polyamines by *M. smegmatis* was found to be inhibited by compounds also inhibiting polyamine oxidation by *P. aeruginosa* (Razin *et al.* 1959). Zeller, Owen & Karlson (1951) found that dihydrostreptomycin inhibited the oxidation of putrescine and other amines by *M. smegmatis*. Their results could not be confirmed by us; even at high concentrations of dihydrostreptomycin the oxidation of putrescine by the same organism was hardly affected. However, dihydrostreptomycin inhibited the oxidation of putrescine by cells of *M. smegmatis* unadapted to this amine (Persky, 1959). These findings suggest that the inhibitory effect of dihydrostreptomycin described by Zeller *et al.* (1951) might be due to inhibition of adaptive amine-oxidase formation, and not a direct effect on oxidation (cf. Geronimus, 1951). The inhibition of adaptive-enzyme formation by streptomycin is well known (Eagle & Saz, 1955).

SUMMARY

1. Spermine and spermidine were oxidized by cells of *Mycobacterium smegmatis*. The oxidation was enhanced by the incorporation of spermine into the culture medium.

2. Spermidine, γ-aminobutyric acid, propane-1:3-diamine and β-alanine were found to be intermediates in the oxidation of spermine by freeze-dried cells.

3. Cells of *M. smegmatis* and *Pseudomonas aeruginosa* degraded γ-aminobutyric acid and β-alanine by transamination with the formation of succinic and malonic semialdehydes. The semialdehydes were further oxidized to succinate and malonate by freeze-dried cells of *M. smegmatis*.

4. Semicarbazide and *iso*nicotinic acid hydrazide inhibited the oxidation of spermine by *M. smegmatis*. Dihydrostreptomycin did not affect the oxidation of putrescine.

5. The possibility of a general pathway for the bacterial degradation of spermine and spermidine is discussed.

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Oxidative Metabolism of Phthalic Acid by Soil Pseudomonads

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Phthalic acid is removed from chemical waste effluents by biological purification through filter beds. This has led to an investigation of the oxidative metabolism of this aromatic compound by the micro-organisms responsible for this action industrially, and from the soil.

In mammals, e.g. dog and man, phthalic acid is largely excreted unchanged, and does not appear to form glycine conjugates (Shemyakin & Schukina, 1944). Jacobs (1931), on very slender evidence, suggested that phthalic acid was an intermediate in the dissimilation of naphthalene by soil micro-organisms; Fernley & Evans (1958) have shown this view to be incorrect.

From young liquid cultures of a soil pseudomonad growing on phthalate, Evans (1955) identified, by chromatography, the presence of 4:5-dihydroxyphthalate. Application of the technique of sequential induction revealed that this acid and protocatechuic acid were immediately metabolized by phthalate-grown cells. The adaptation to protocatechuic acid was not conclusive evidence of its being an intermediate, since it might have arisen from *p*-hydroxybenzoic acid, a known anabolic product of most bacteria. Further, a hitherto undescribed enzyme reaction (a non-oxidative aromatic decarboxylation) would have been implied. We have extended this study, a preliminary report of which has been presented (Ribbons & Evans, 1959*a*, *b*).

EXPERIMENTAL

Isolation of organism. Microbes isolated from filter beds actively detoxicating phthalic acid at Monsanto Chemicals, Ruabon, were kindly made available through the courtesy of Dr G. Wilson and Miss M. Harrison. Our own isolates were obtained by the elective-culture method from garden soil, manure and coniferous litter. The enrichment medium, adjusted to pH 7 with NaOH, contained the following (g./l.): phthalic acid, 1; $(NH_4)_2SO_4$, 1; $MgSO_4$, $7H_2O$, 0·5;

NaCl, 0·25; $CaCl_2$, 0·25; $FeCl_2$, 0·02; $MnSO_4$, 0·002; $ZnSO_4$, 0·002; $CuSO_4$, 0·002. Pure cultures were obtained after a few transplants on phthalate–mineral salts–agar plates. Most of the isolates were Gram-negative motile organisms with characteristics placing them in the family Pseudomonadaceae; one strain, isolated from conifer litter, was selected for metabolic work. The organism was maintained by serial subculture on phthalate–mineral salts–agar slopes.

Cultural methods and harvesting of cells. Liquid cultures were grown in 10 l. Pyrex bottles, medium of the above composition being used with forced aeration with sterile air, either at room temperature or in a bath at 25°. Maximum growth, ascertained by turbidity measurements, was obtained within 4–5 days. Cells were harvested by centrifuging at 13 000 *g* in a bench-model Sharples super-centrifuge, furnished with a cellophan lining to the rotor; they were washed twice with 0·1 M-phosphate buffer and stored at −15° until required. Unadapted cells were grown on 0·1% glucose–mineral salts medium, the mineral base being identical with that used in the phthalate medium.

Preparation of cell-free extracts. Washed cells (1 g. wet wt.) were suspended in 5 ml. of water or of buffer of choice, and disintegrated either in a Mickle apparatus for 15 min. with Ballotini glass beads, grade 12 (English Glass Co., Leicester), or sonically with no abrasive in the M.S.E.–Mullard Ultrasonic Disintegrator operating between 16 and 24 kcyc./sec. at 0–4°; or with the Hughes press (Hughes, 1951) without abrasive. The suspension was centrifuged at 1000 *g* for 10 min. to remove unbroken cells and debris, and the supernatant was again spun at 20 000 *g* in the super-speed angle head of the M.S.E. refrigerated centrifuge for 20–30 min. to remove smaller particulate matter in the extract.

Materials. 4:5-Dihydroxyphthalic acid was synthesized by the demethylation of *m*-hemipinic acid [obtained by the method of Perkin, Edwards & Stoyle (1925) from veratraldehyde] with hydriodic acid (sp.gr. 1·73).

3:4-Dihydroxyphthalic acid was prepared by demethylation of hemipinic acid (L. Light and Co. Ltd.) with PCl_5 at 180°, and boiling the resulting 3:4-bischloromethoxyphthalic anhydride with water (Freund & Horst, 1894).

Protocatechuic acid was synthesized by the method of Pearl (1949).

3-Hydroxyphthalic acid was prepared by the method of Pratt & Perkins (1918).

4-Hydroxyphthalic acid was prepared by the method of Bentley, Robinson & Weizmann (1907).

2:4:5-Trihydroxybenzoic acid was synthesized from 2:4-dihydroxyacetophenone by treatment with iodine in pyridine and subsequent treatment with alkali (Thiele & Jaegar, 1907).

cis-cis-Muconic acid and γ-carboxymethyl-Δ^{α}-butenolide were prepared by the methods of Elvidge *et al.* (1950).

Oxythiamine diphosphate was synthesized by the method of Cerecedo & Eusebi (1950).

Other chemicals were available commercially; all melting points are uncorrected.

Estimations. Phenols were determined by the Folin–Ciocalteu reagent and *o*-dihydroxyphenols by the Evans (1947) reagent.

Spectrophotometric determinations were carried out in the Unicam SP. 500 in 1 cm. light-path silica cuvettes. Protein was estimated by the method of Warburg & Christian (1942). Protocatechuic acid was estimated spectrophotometrically (Stanier & Ingraham, 1954).

Manometric experiments were made in the conventional Warburg constant-volume apparatus at 30°. Oxygen uptake and evolution of CO_2 were estimated by the direct method (Dixon, 1952). 3-Oxoadipic acid was estimated by catalytic decarboxylation with 4-aminoantipyrine (Sistrom & Stanier, 1953). The enzyme system that decarboxylates 4:5-dihydroxyphthalate to protocatechuate (4:5-dihydroxyphthalate decarboxylase) was conveniently assayed by evolution of CO_2 under anaerobic conditions. The standard reaction mixture for the assay was: 4:5-dihydroxyphthalate (10 μmoles in a volume of 0·2 ml. in the side arm), 0·1 M-phosphate buffer, pH 6·5 (2 ml.), enzyme solution (concentration being such that between 5 and 50 μl. of CO_2 was liberated in 5 min.) and water to make a final volume of 3 ml. The flasks were equilibrated for 10 min., during which they were flushed with CO_2+N_2 (5:95) gas mixture. The last traces of O_2 were removed from the manometer arm by manipulation of the manometer fluid. The reactions were initiated by tipping the substrate into the main compartment and initial velocities taken as the measure of activity. Enzyme activity is expressed as μmoles of CO_2 liberated/mg. of protein/hr.

Chromatography. Both ascending and descending techniques were used with Whatman no. 4 paper; all chromatograms were run at room temperature. The two solvents used were benzene–acetic acid–water (80:20, saturated) and butanol–acetic acid–water (40:50:10). The R_F values given in the former solvent have been published (Coulson & Evans, 1958); better separation of the dihydroxyphthalic acids and protocatechuic acid was obtained with the latter solvent, the R_F values being 0·52, 0·48 and 0·73 for 3:4-dihydroxyphthalic acid, 4:5-dihydroxyphthalic acid and protocatechuic acid respectively.

Detection reagents. Phenolic substances were detected by spraying the air-dried chromatograms with the following reagents: (*a*) diazotized *p*-nitroaniline or sulphanilic acid, followed by sodium carbonate solution (Bray & Thorpe, 1954), or (*b*) Folin–Ciocalteu reagent, followed by exposure of the chromatogram to NH_3 fumes (Mitchell & Davies, 1954).

o-Dihydroxyphenolic substances were detected by either ammoniacal silver nitrate or the molybdate–nitrite sprays (Coulson & Evans, 1958).

Acids were detected by spraying with bromocresol green (Lugg & Overall, 1948).

Ultraviolet-absorbing materials were detected with a Hg lamp (253·7 mμ).

Buffers. Incubations were carried out in either Na_2HPO_4–KH_2PO_4 buffer or 2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris–HCl) buffer. The proportions of each constituent were varied according to the pH required and the molarity of the resulting buffer was as indicated in the text.

RESULTS

Sequential-induction experiments

Washed cells harvested in the logarithmic phase of growth on phthalate or glucose were used. Cells grown upon phthalate immediately oxidized phthalate, 4:5-dihydroxyphthalate and protocatechuate without any appreciable induction period

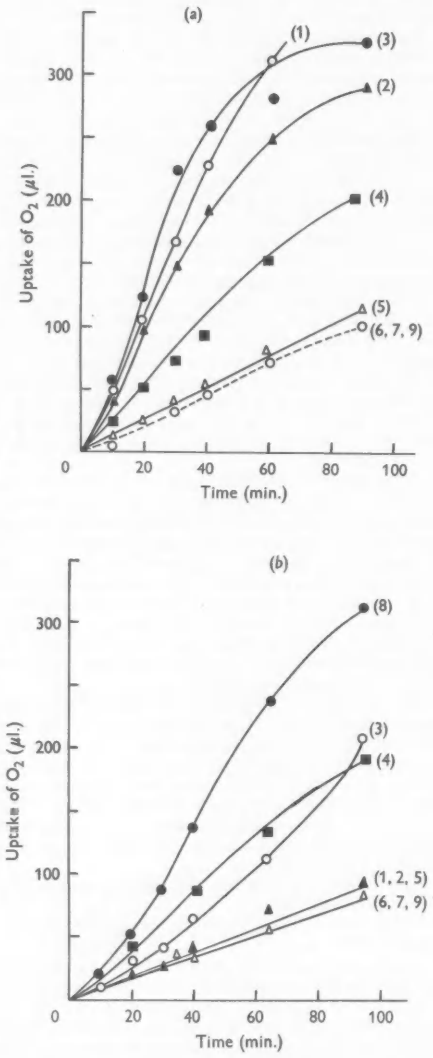

Fig. 1. Oxidation of various compounds by whole cells grown (*a*) with phthalate and (*b*) with glucose. Each Warburg flask contained: bacterial suspension (approx. 10 mg. dry wt. in 0·065 M-phosphate buffer, pH 7) 1 ml.; 0·067 M-phosphate buffer, 1·8 ml. Side arms contained 5 µmoles of the following substrates dissolved in 0·2 ml. of the same buffer. The centre well contained 0·2 ml. of 20% KOH. Temperature, 30°. Atmosphere, air. (1) Phthalate; (2) 4:5-dihydroxyphthalate; (3) protocatechuate; (4) succinate; (5) 3:4-dihydroxyphthalate; (6) 3-hydroxyphthalate; (7) 4-hydroxyphthalate; (8) glucose; (9) no substrate.

(Fig. 1). Succinate was also oxidized, being included in these experiments as a typical member of the tricarboxylic acid cycle. These cells were not sequentially induced to the following substrates: 3-hydroxyphthalate, 4-hydroxyphthalate, *o*-, *m*- and *p*-hydroxybenzoate, phenol, catechol, the dihydroxybenzoates (except protocatechuate), 3:4-dihydroxyphthalate, 2:4:5-trihydroxybenzoate, *cis-cis*-muconate or γ-carboxymethyl-Δ^{α}-butenolide.

Glucose-grown cells did not metabolize any of these aromatic compounds immediately, indicating that the enzymes responsible for their dissimilation are induced (Fig. 1).

Detection of phenolic intermediate in growing cultures

Early in the logarithmic phase of growth, some phthalate-grown cultures gave transiently an *o*-dihydroxyphenol reaction, detected by the Evans reagent. This is illustrated for a typical culture in Fig. 2.

When the *o*-dihydroxyphenol reaction was at a maximum, a 10 l. culture was centrifuged, acidified to Congo red with 3 N-HCl, concentrated under reduced pressure to 400 ml. and extracted with ether (three times with 200 ml. portions). The combined ether extracts were dried over Na_2SO_4; evaporation of the ether at 35°/760 mm. Hg yielded 1·5 g. of yellow crystals. Chromatography of this material revealed two components detected by u.v. light and by bromocresol-green indicator. Their R_F values were identical with protocatechuic acid and phthalic acid in both solvent systems. Further support for the identity of the phenolic component was obtained by the application of the phenolic and *o*-dihydroxyphenolic sprays. Determination of the u.v.-absorption spectra of the

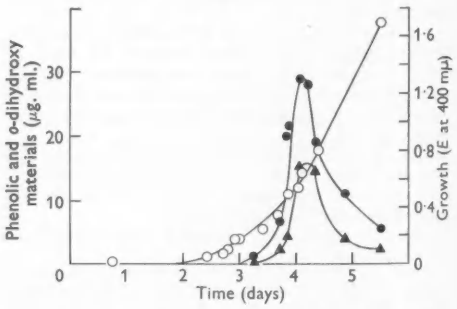

Fig. 2. Accumulation of phenolic and *o*-dihydroxy materials in a typical phthalate culture (10 l.). ○, Growth (measured by measurements of *E* at 400 mµ); ●, phenolic materials produced; ▲, *o*-dihydroxyphenolic materials produced.

eluted spots showed them to be identical with authentic samples of phthalic acid and protocatechuic acid (Fig. 3).

Oxidation with acetone-dried powders and cell-free extracts

4:5-Dihydroxyphthalic acid and protocatechuic acid were rapidly oxidized by acetone-dried powders of washed cells grown on phthalate. Phthalic acid oxidation is much reduced by this treatment and none of the other aromatic substrates mentioned was metabolized (Fig. 4*a*).

Cell-free extracts, prepared by any of the methods outlined above, readily oxidized 4:5-dihydroxyphthalate, protocatechuate and succinate. None of these extracts, either alone or with the addition of cell debris, oxidized phthalate, 3-hydroxy- or 4-hydroxy-phthalate, or any of the substrates previously listed in the sequential-induction experiments.

4:5-Dihydroxyphthalate is metabolized with the uptake of 1 mole of O_2 and evolution of 2 moles of CO_2 per mole of substrate; with protocatechuate 1 mole of O_2 was consumed with evolution of 1 mole of CO_2 per mole of substrate (Fig. 4*b*). The end product in each case is 3-oxoadipate, detected by the Rothera reaction (Rothera, 1908) and

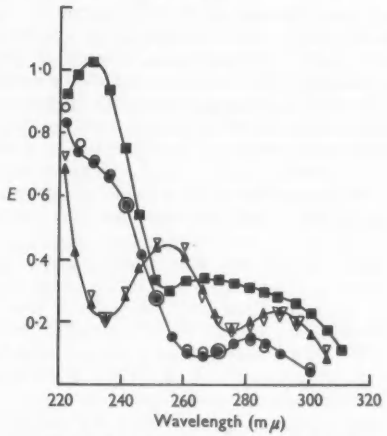

Fig. 3. Ultraviolet-absorption spectra of phthalic acid (●), 4:5-dihydroxyphthalic acid (■) and protocatechuic acid (▲). Each compound was dissolved in 0·1 M-phosphate buffer, pH 7, to give a concentration of 0·03–0·04 mM. The spectra of the eluted chromatogram spots, obtained from ether extraction of cultures, are also shown; from the butanol–acetic acid–water solvent the spot of R_F 0·8 (identical with that of phthalic acid) (○); and spot of R_F 0·7 (identical with that of protocatechuic acid) (▽).

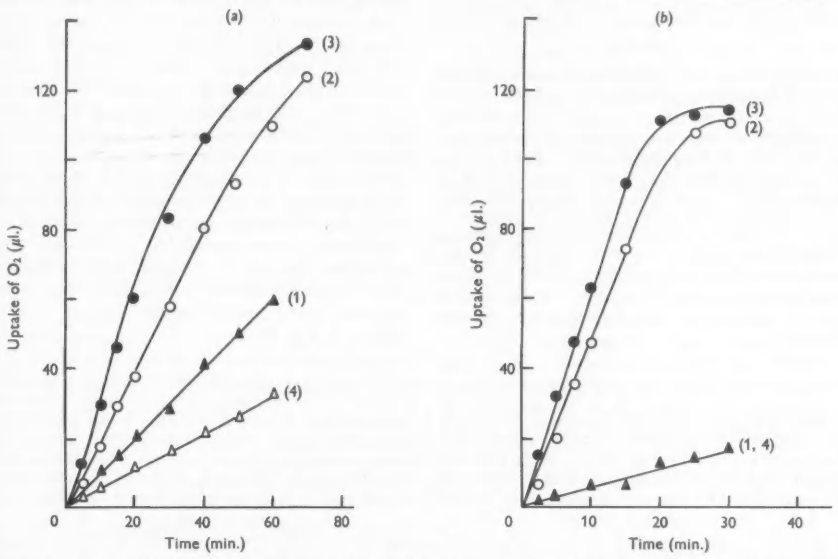

Fig. 4. Oxidation of various compounds by acetone-dried powders and cell-free extracts. Each Warburg flask contained: 0·067 M-phosphate buffer, pH 7, 1·8 ml., and either (*a*) 1 ml. of acetone-dried cells (approx. 10 mg. dry wt. in 0·067 M-phosphate buffer, pH 7) or (*b*) 1 ml. of cell-free extract prepared in the Mickle disintegrator (14 mg. of protein in the same buffer). Side arms contained 5 μmoles of the following substrates dissolved in 0·2 ml. of the same buffer (0·2 ml.). The centre well contained 0·2 ml. of 20% KOH. Temperature, 30°. Atmosphere, air. (1) Phthalate; (2) 4:5-dihydroxyphthalate; (3) protocatechuate; (4) no substrate.

estimation by catalytic decarboxylation. For each mole of either 4:5-dihydroxyphthalate or protocatechuate, 1 mole of 3-oxoadipate is formed (Table 1). This suggests that 4:5-dihydroxyphthalate is first decarboxylated to protocatechuate, before ring-cleavage. This decarboxylation may be accomplished under anaerobic conditions. The relative rates of decarboxylation and oxidation of 4:5-dihydroxyphthalate under aerobic conditions are shown in Fig. 5 and are consistent with the view that 4:5-dihydroxyphthalate decarboxylation is an intermediate step in its overall oxidation by crude extracts.

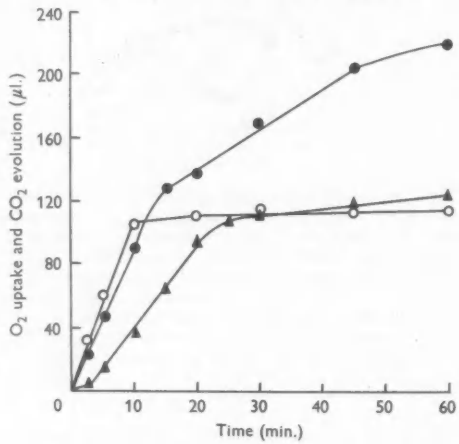

Fig. 5. Relative rates of decarboxylation and subsequent oxidation of 4:5-dihydroxyphthalate by cell-free extracts under aerobic and anaerobic conditions. Each Warburg flask contained 4:5-dihydroxyphthalate (5 μmoles in a volume of 0·2 ml.); 0·1 M-phosphate buffer, pH 6·0, 2·0 ml.; cell-free extract (containing 15 mg. of protein), 1 ml. Temperature, 30°. Atmosphere was air for the direct method of estimation of O_2 uptake and CO_2 evolution, and CO_2+N_2 (5:95) for the anaerobic decarboxylation of 4:5-dihydroxyphthalate. ▲, O_2 uptake; ●, CO_2 evolution (direct method, aerobic); ○, CO_2 evolution (anaerobic, see Experimental section). Points are all corrected for oxidation and decarboxylation in the absence of substrate.

4:5-*Dihydroxyphthalate decarboxylase*

Conversion of 4:5-*dihydroxyphthalate into protocatechuate.* The evolution of an equimolar amount of CO_2 on anaerobic incubation of crude extracts with 4:5-dihydroxyphthalate suggested a conversion of the substrate into protocatechuate. The change in a u.v.-absorption spectrum supported such an enzyme action, and protocatechuate was isolated from the reaction mixture as follows.

To each of thirty-six Warburg flasks (20 ml. capacity) were added: 4:5-dihydroxyphthalate (10 μmoles in a volume of 0·2 ml.) in the side arm; 0·1 M-phosphate buffer, pH 6·5 (2 ml.), and cell-free extract (1 ml., approx. 7 mg. of protein). The flasks were equilibrated at 30° under CO_2+N_2 (5:95) and the reaction was followed as described in the Experimental section. To each flask was added 2 N-HCl (0·2 ml.) after completion of the CO_2 evolution and the flask contents were bulked. After, the contents were centrifuged and the supernatant was extracted three times with an equal volume of ether. The combined ether extracts were dried over Na_2SO_4 and distilled to dryness at 35°. An off-white crystalline material (45 mg.) remained, which melted at 185–191°. This was decolorized with activated charcoal and recrystallized twice from water. The crystals so obtained melted at 197–198° and, upon admixture with authentic protocatechuic acid (m.p. 198–199°), melted at 197–198°. All chromatographic, chemical and absorption characteristics were consistent with the material being protocatechuic acid.

Properties of 4:5-*dihydroxyphthalate decarboxylase.* The enzymic decarboxylation of 4:5-dihydroxyphthalate to protocatechuate was assayed manometrically under anaerobic conditions by CO_2 evolution, the standard conditions described in the Experimental section being used. The effect of enzyme concentration upon rate of reaction is shown in Fig. 6.

Table 1. *Stoicheiometry of* 4:5-*dihydroxyphthalate and protocatechuate oxidations by crude extracts*

Each flask contained: 0·2 M-phosphate buffer, pH 7, 1 ml.; extract (13 mg. of protein), 1 ml.; substrate in side arm, 0·2 ml. (neutralized to pH 7, amount as indicated) and water to make a total volume of 3 ml. The centre well contained 0·2 ml. of 20% KOH for measurements of O_2 uptake. Temperature, 30°. Incubation time, 60 min. Total CO_2 evolution was estimated by tipping in acid (0·2 ml., 2 N-HCl) from double-side-arm flasks and the values listed are corrected appropriately for retention with similar flasks containing no substrate.

Expt. no.	Substrate	Substrate (μmoles)	Uptake of O_2 (μmoles)	Evolution of CO_2 (μmoles)	3-Oxoadipate formed (μmoles)
1	4:5-Dihydroxyphthalate	3	2·91	5·9	2·58
2		6	5·9	11·7	5·6
3	Protocatechuate	3	3·05	3·1	2·75
4		6	5·96	6·1	5·65

The crude extract may be stored at $-15°$ for up to 3 months, in either water or phosphate buffer (0·067 M, pH 6·5), without appreciable loss of activity, and at 5° for 2–3 days. Protocatechuic acid and CO_2 were the only products detected, and the reaction was essentially irreversible under these conditions, the stoicheiometry being shown in Table 2.

The optimum pH was between 6·0 and 6·7 in phosphate buffer and substitution by tris–HCl buffer had no effect upon activity. Dialysis against distilled water or phosphate buffer (0·067 M) over a range of pH 5·9–8·5, at 0–3°, resulted in no appreciable loss of activity. Prolonged dialysis (3–4 days) caused a loss of about 70 % of original activity; this was not restored by addition of boiled enzyme or of thiol compounds or both. Supplements of cocarboxylase, Mg^{2+}, Mn^{2+}, reduced glutathione (GSH), coenzyme A (CoA) and adenosine triphosphate (ATP) at concentrations up to 1 mM to the dialysed residues or cell-free extracts did not increase the activity in any combination tried; in fact CoA, ATP and cocarboxylase were slightly inhibitory. No intermediates of the reaction were detected with hydroxylamine as a trapping agent.

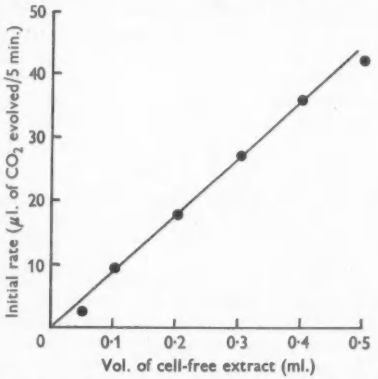

Fig. 6. Effect of enzyme concentration on 4:5-dihydroxyphthalate decarboxylase. Each flask contained: 4:5-dihydroxyphthalic acid (10 µmoles in a volume of 0·2 ml. in side arm); 0·1 M-phosphate buffer, pH 6·5, 2 ml.; cell-free extract (15 mg./ml.) as indicated and water to give a final volume of 3 ml. Atmosphere, CO_2+N_2 (5:95). Temperature, 30°.

Table 2. *Stoicheiometry of 4:5-dihydroxyphthalate decarboxylase*

Each double-arm flask contained the standard reaction mixture with 1 ml. of cell-free extract containing 13 mg. of protein (prepared in the Mickle disintegrator), except that the amount of substrate was as indicated and 2 N-HCl (0·2 ml.) was added to the second side arm. The acid was tipped in at the termination of CO_2 evolution to prevent any of the formed protocatechuate being oxidized enzymically on exposure to air. The acidified mixtures were centrifuged and samples taken to estimate the protocatechuate formed spectrophotometrically. A flask containing all reagents except substrate was used for a blank in the spectrophotometer.

Expt. no.	Substrate (µmoles)	CO_2 evolved (µmoles)	Protocatechuate formed (µmoles)
1	5	4·82	4·75
2	10	9·67	9·6

4:5-Dihydroxyphthalate decarboxylase was partially purified from crude extracts by $(NH_4)_2SO_4$ fractionation. All operations were conducted between 2 and 5°. To the crude extract in 0·067 M-phosphate buffer, pH 7·0 (100 ml.), prepared in the Mickle disintegrator, was added saturated $(NH_4)_2SO_4$ (adjusted to pH 7·0 with NH_3 soln.) up to 33 % saturation, and, after standing for 30 min., the precipitate was centrifuged. The supernatant was brought to 66 % saturation by further addition of saturated $(NH_4)_2SO_4$ solution and the flocculent precipitate collected and redissolved in water (100 ml.). Refractionation of this with saturated $(NH_4)_2SO_4$ up to 40, 50 and 66 % showed that most of the activity was present in the 40–50 % fraction (Table 3). This fraction was dissolved in water (50 ml.) and used for inhibition experiments (Table 4).

Oxidation of protocatechuate by cell-free extracts

Oxygen uptake and evolution of CO_2 are consistent with the oxidation of protocatechuate to 3-oxoadipate, by extracts incubated with protocatechuate. This was confirmed by the isolation of 3-oxoadipic acid as the reaction product. The twice-recrystallized material had m.p. 118–119° and the mixed m.p. with an authentic sample of 3-oxoadipic acid (m.p. 120–121°) was 118·5–120°.

Dialysis of the crude extracts against tap water at 0–3° for 60 hr. did not suppress protocatechuic acid oxidation, but stopped the sequence of reactions at some point before 3-oxoadipate. The product formed has the u.v. spectrum of *cis-cis-β*-carboxymuconic acid (MacDonald, Stanier & Ingraham, 1954). This was confirmed by the isolation of the trisodium salt, as described previously by MacDonald *et al.* (1954). Incubation of the isolated *cis-cis-β*-carboxymuconate with undialysed extracts yielded 3-oxoadipate, as detected by the Rothera reaction and identification as the 2:4-dinitrophenylhydrazone of laevulinate (Kilby, 1951).

DISCUSSION

The reaction sequence as illustrated in Fig. 7 would account for the observed results.

Whole cells grown on phthalic acid as sole carbon source readily oxidized this substrate, but

Table 3. *Summary of 4:5-dihydroxyphthalate-decarboxylase purification by precipitation with ammonium sulphate*

4:5-Dihydroxyphthalate decarboxylase was assayed under standard conditions as described in the Experimental section.

Fraction	Concn. of protein (mg./ml.)	Activity (μmoles of CO_2/mg. of protein/hr.)
Crude Mickle cell-free extract	16·4	2·46
(1) $(NH_4)_2SO_4$ ppt. (33–66%, w/v, saturated)	8·45	4·42
(2) $(NH_4)_2SO_4$ ppt. prepared from (1) (35–50%, w/v, saturated)	3·8	11·5

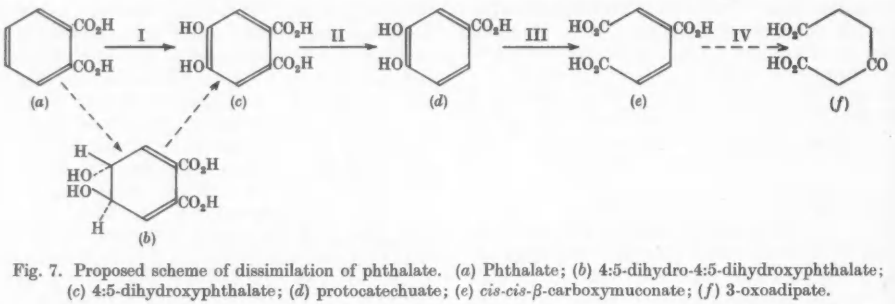

Fig. 7. Proposed scheme of dissimilation of phthalate. (*a*) Phthalate; (*b*) 4:5-dihydro-4:5-dihydroxyphthalate; (*c*) 4:5-dihydroxyphthalate; (*d*) protocatechuate; (*e*) *cis-cis-*β-carboxymuconate; (*f*) 3-oxoadipate.

Table 4. *Effect of various inhibitors on 4:5-dihydroxyphthalic acid decarboxylase*

Each Warburg cup contained the standard reaction mixture (see Experimental section) with inhibitors as indicated, the partially purified enzyme being used (950 μg. of protein per flask). EDTA, Ethylenediaminetetra-acetic acid.

Compound (addition)	Concn. (mM)	Inhibition (%)
KF	1	24·5
8-Hydroxyquinoline	1	0
EDTA	1	87·9
EDTA	0·6	54
EDTA	0·3	35
EDTA + $MgSO_4,7H_2O$ (3 mM)	0·3	27
EDTA + $MnSO_4,4H_2O$ (3 mM)	0·3	13
Iodoacetate	2	84
Cystine	1	20
p-Chloromercuribenzoate	1	100
$HgCl_2$	1	100
Phenyl mercuric nitrate	1	100
Oxythiamine diphosphate	1	0

crude cell-free extracts, prepared by the methods described, were unable to do so. A factor involved might have been removed or diluted out during their preparation, since relatively dilute suspensions of the cells were disintegrated (see Dagley & Patel, 1957).

Neither the 3-hydroxyphthalate nor the 4-hydroxyphthalate was metabolized by whole cells grown on phthalate; this might mean that either a permeability barrier exists which prevents these substances from reaching the appropriate intracellular-enzyme sites, or that there is an absence of such enzymes. Cell extracts, prepared by a variety of different methods, were also inactive on the monohydroxyphthalates. Acetone-dried cell powders could still oxidize phthalate (although at a much decreased rate compared with whole cells) but were without any action on the monohydroxyphthalates. These observations exclude both the monohydroxyphthalates as intermediates in phthalate metabolism. We therefore suggest that 4:5-dihydro-4:5-dihydroxyphthalate may well be formed initially, by perhydroxylation of phthalate, followed by dehydrogenation to 4:5-dihydroxyphthalate. A test of this hypothesis awaits the preparation of the relevant diol. A similar reaction is believed to be of widespread occurrence in the bacterial and mammalian metabolism of many aromatic compounds (Williams, 1950; Parke & Williams, 1958; Evans, 1956, 1957; Mason, 1957).

The decarboxylation of 4:5-dihydroxyphthalate to protocatechuate was the earliest reaction in the sequence we could demonstrate by cell-free extracts. This simple decarboxylation, directly from the benzene nucleus, is a new enzymic reaction in aromatic-ring biochemistry. Other decarboxylations from the ring previously reported have been oxidative in nature, e.g. salicylate (Walker & Evans, 1952; Murphy & Stone, 1954), benzoate

(Sleeper, 1951) and 6-hydroxynicotinate (Stanier & Behrmann, 1957). The decarboxylation of anthranilic acid to aniline (McCullough, Piligian & Daniel, 1957) is pyridoxal phosphate-linked.

Attempts to determine the cofactor requirements for this new enzyme did not meet with much success. The inactivity of CoA, ATP, cocarboxylase and pyridoxal phosphate with crude and dialysed extracts could have been due to the presence of phosphatases, which were detected in the extracts. However, the inhibition by ethylenediaminetetra-acetic acid and subsequent partial reversal by Mn^{2+} and Mg^{2+} ions suggest that a bivalent ion may be required for optimum activity, in addition to essential thiol groups of the enzyme. Attempts to trap intermediates in the decarboxylation, with hydroxylamine, were negative, suggesting that phosphate or thio-esters are not involved.

The isolation of 3-oxoadipate and *cis-cis-β*-carboxymuconate from protocatechuate oxidation by the use of cell-free extracts and diffusates respectively are in accordance with the work of MacDonald *et al.* (1954).

Dialysis of the extracts did not suppress protocatechuate oxidation, indicating that there is no easily dissociable cofactor involved in this case. This was also found by Stanier & Ingraham (1954). Other systems which perform similar oxygenations, e.g. pyrocatechuase (Suda, Hashimoto, Matsuoka & Kamahora, 1951), homogentisic acid oxidase (Ravdin & Crandall, 1951), gentisic acid oxidase (Lack, 1959), 3-hydroxyanthranilic acid oxidase (Wiss, 1957), *Neurospora* protocatechuic acid oxidase (Ottey & Tatum, 1956) and the *Pseudomonas* protocatechuic acid oxidase of Dagley & Patel (1957), have all been shown to require Fe^{2+} ions for optimum activity. The enzyme in Stanier's and our systems may well be Fe^{2+} ion-dependent, but we could not demonstrate stimulation by Fe^{2+} ions in extracts or dialysed extracts. We presume that Fe^{2+} is very tightly bound to the apo-enzyme.

The conversion of *cis-cis-β*-carboxymuconic acid into 3-oxoadipate by *Pseudomonas* still requires further elucidation. Ribbons & Evans (1959*b*) have shown that 2:6-dioxa-3:7-dioxo-*bicyclo*[3:3:0]-octane (dilactone) is converted into 3-oxoadipate by extracts of cells grown on phthalate. Such a symmetrical intermediate or its open-chain counterpart *ββ′*-dihydroxyadipic acid is required from the isotopic evidence of Gross, Gafford & Tatum (1956). Whether the dilactone is a true intermediate has yet to be determined.

SUMMARY

1. Protocatechuic acid was detected and identified early in the logarithmic phase of growth in phthalate cultures of *Pseudomonas* sp.

2. Washed whole cells were sequentially induced to phthalate, 4:5-dihydroxyphthalate and protocatechuate.

3. Cell-free extracts catalysed the conversion of 4:5-dihydroxyphthalate, protocatechuate and *cis-cis-β*-carboxymuconate into 3-oxoadipate. The amounts of gaseous exchange supported data from experiments with whole cells, that the compounds participated in a reaction sequence in that order.

4. Extracts incubated with 4:5-dihydroxyphthalate under anaerobic conditions produced equimolar amounts of carbon dioxide and protocatechuate. After a fivefold purification of 4:5-dihydroxyphthalic acid decarboxylase, the enzyme showed inhibition by sulphydryl-group reagents and ethylenediaminetetra-acetic acid; the latter effect was partially reversed by manganese and magnesium ions.

5. The oxidation of protocatechuate by these extracts proceeded through *cis-cis-β*-carboxymuconate and 3-oxoadipate, which were isolated. No requirement for ferrous ions could be detected with this protocatechuic acid oxidase.

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Synthesis of Optical Isomers of Cystinylvaline

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It has been shown (Arnstein & Grant, 1954*a*, *b*; Arnstein & Crawhall, 1957) that L-cystine, presumably after reduction to cysteine, is incorporated intact into the β-lactam–thiazolidine nucleus of penicillin; L-valine can also be utilized without deamination or transamination in the biosynthesis of the nucleus (Stevens & De Long, 1958).

The elucidation of the mode of formation of the β-lactam–thiazolidine ring system from cystine and valine is of considerable interest and it has been suggested (Arnstein, 1957) that cysteinylvaline or its disulphide form may function as a precursor. The present paper deals with the synthesis of several optical isomers of cystinylvaline, including two labelled with ^{14}C in the valine carboxyl group.

The main purpose of the synthesis with unlabelled cystine and valine was to find methods which would yield L-cystinyl-L-valine and L-cystinyl-D-valine in an optically pure state. A number of diastereoisomeric amino acids and peptides have been separated by ion-exchange chromatography on Dowex 50 (Blackburn & Lee, 1955; Blackburn & Tetley, 1956; Blackburn & Schöberl, 1956), and it was thought possible that L-cystinyl-DL-valine could be separated into its three isomers in this way. Accordingly, this peptide was synthesized by the route shown in Fig. 1; benzyloxycarbonyl-*S*-benzyl-L-cysteine was coupled with DL-valine ethyl ester by the mixed-anhydride method of Vaughan (1951) and, after saponification of the resulting protected dipeptide ester, both the benzyloxycarbonyl and *S*-benzyl groups were removed simultaneously with sodium in liquid ammonia (pathway B, Wood & Du Vigneaud, 1939). Crystallization of the crude product, as described in the Experimental section, gave, surprisingly, optically pure L-cystinyl-D-valine. The mother liquors were evaporated to dryness and the residue was placed on a column of Zeo-Karb 225 (The Permutit Co. Ltd., London) and subjected to gradient elution with hydrochloric acid (0·5–3·0N); at an acid concentration of 2·6N a component, running as one spot in solvent A, was obtained which, after hydrolysis and separation of the cystine and valine, was shown to be L-cystinyl-DL-valine. No evidence for the separation of L-cystinyl-L-valine from the mixture by this method was found. This approach to the synthesis of the two peptides was therefore abandoned. L-Cystine was found to be racemized to a considerable extent under conditions of acid hydrolysis; the optical configurations of the cystine and valine, isolated after hydrolysis of the peptides, were determined by comparison of their specific rotations with those of cystine and valine obtained from synthetic mixtures of optically pure amino acids, which had undergone identical treatment with acid. Cystinylvaline is fairly resistant to hydrolysis, complete reaction requiring 64 hr. with 6N-hydrochloric acid at 110°.

Synthesis of the two required peptides was commenced with L-cystine and either L- or D-valine. The coupling method with di*cyclo*hexylcarbodi-

imide (Sheehan & Hess, 1955) was successful; yields of 80–90 % were obtained. Removal of the protecting benzyloxycarbonyl and *S*-benzyl groups, after saponification of the fully protected dipeptide esters, however, gave some difficulty. It was found that removal of the two groups simultaneously with sodium in liquid ammonia gave rise to considerable racemization of the cystine moiety, the free peptide exhibiting several spots on paper chromatography even though the valine was optically pure. This difficulty was largely overcome by removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid before reduction in liquid ammonia (pathway A). Even so, racemization of cystine did occur in the reduction of *S*-benzyl-L-cysteinyl-D-valine. It is essential that the reduction is carried out under absolutely anhydrous conditions with fresh bright sodium and liquid ammonia that has been dried by distillation from sodium in a closed system.

Synthesis of the peptides labelled with ^{14}C in the valine carboxyl group was achieved via the *cyclo*hexylcarbodi-imide-coupling method, the protecting groups being removed by pathway A (Fig. 1). Desalting of the peptides after the reduction was facilitated to some extent by the use of lithium instead of sodium, since lithium bromide, formed on addition of ammonium bromide at the end of the reduction, is soluble in ethanol and was largely removed from the peptide–salt mixture by extraction of the latter with hot ethanol. The overall conversion of L-[*carboxy*-^{14}C]valine and D-[*carboxy*-^{14}C]valine into the corresponding radiochemically pure peptides was 35 and 31 % respectively.

A preliminary account of some of this work was given by Arnstein & Morris (1959).

EXPERIMENTAL

Radioactivity measurements. Compounds were mounted on disks 1 cm.^{2} and counted at infinite thickness, a thin end-window Geiger–Müller tube being used (Popják, 1950). The specific radioactivities were obtained by direct comparison with a poly[^{14}C]methyl methacrylate standard (1 μc/g.) obtained from The Radiochemical Centre, Amersham, Bucks. The standard error in all determinations is not greater than 5 %.

Paper chromatography. All R_F values refer to descending chromatograms on Whatman no. 1 paper, unless otherwise stated. The solvent systems had the following composition (by vol.): A, butanol–acetic acid–water, 63:10:27; B, amyl alcohol–pyridine–water, 7:7:6; C, phenol–water, 5:2 (with NH_3).

Synthesis of peptides. The routes to the peptides are outlined in Fig. 1. The descriptions L-DL, etc. refer to the optical configurations of the cystine and valine portions of the dipeptide respectively. All melting points are uncorrected.

Synthesis involving separation of diastereoisomers

Benzyloxycarbonyl-*S*-benzyl-L-cysteine (II; 6·0 g.), prepared from L-cystine via *S*-benzyl-L-cysteine (Wood & Du Vigneaud, 1939), was dissolved in dry tetrahydrofuran (50 ml.) and triethylamine (1·78 g.; 1 mol.prop.) was added. After cooling to −5°, *iso*butylchloroformate (2·4 g.; 1 mol.prop.) was added and the mixture was shaken at −5° for 8 min.

Fischer–Speier esterification of DL-valine (11·7 g.) gave *DL-valine ethyl ester hydrochloride* (III) (18·1 g., 100 %), m.p. 107–108° (Found: C, 46·5; H, 8·9; N, 8·0. $C_7H_{16}ON_2Cl$ requires C, 46·3; H, 8·8; N, 7·7 %). This ester hydrochloride (3·15 g.) was suspended in dry tetrahydrofuran (20 ml.), triethylamine (1·78 g., 1 mol.prop.) was added and the suspension was added to the mixed anhydride solution described above. The mixture was refluxed (1 min.), cooled and poured into aq. 2 % (w/v) $NaHCO_3$ (400 ml.). After standing for 48 hr. at room temperature the solid product was filtered off, dissolved in ethyl acetate (100 ml.) and washed successively with N-HCl (25 ml.), aq. saturated $NaHCO_3$ (2 × 10 ml.) and water. Benzene was added and the solvent was evaporated *in vacuo*; crystallization of the resulting gum from ethyl acetate–light petroleum (b.p. 100–120°) gave *benzyloxycarbonyl-S-benzyl-L-cysteinyl-DL-valine ethyl ester* (IV*a*; R = C_2H_5) (5·0 g., 61 %), m.p.

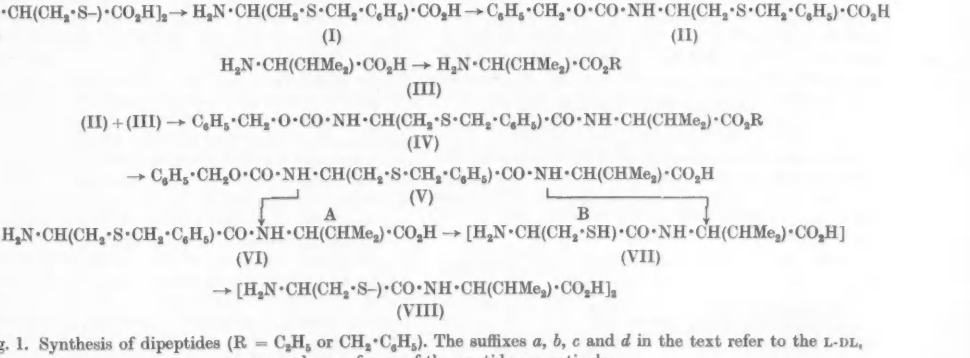

Fig. 1. Synthesis of dipeptides (R = C_2H_5 or $CH_2{\cdot}C_6H_5$). The suffixes *a*, *b*, *c* and *d* in the text refer to the L-DL, LD. LL and DL-D forms of the peptide respectively.

109–113°, $[\alpha]_D^{20}$ – 23·7° (*c*, 2·01 in dioxan) (Found: C, 63·8; H, 6·7; N, 6·2. $C_{25}H_{32}O_5N_2S$ requires C, 63·6; H, 6·8; N, 5·9%). The R_F in solvents A and B was 0·95.

A similar preparation with DL-valine benzyl ester (III, R = $CH_2 \cdot C_6H_5$) (Ben-Ishai & Berger, 1952) gave *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*DL*-valine benzyl ester* (IV*a*; R = $CH_2 \cdot C_6H_5$), m.p. 98–99° (Found: C, 67·3; H, 6·3; N, 5·4. $C_{30}H_{34}O_5N_2S$ requires C, 67·4; H, 6·4; N, 5·2%). The R_F in solvent A was 0·97.

The protected dipeptide ester (IV*a*; R = C_2H_5) (4·65 g.) was dissolved in dioxan (10 ml.) by warming. After cooling to room temperature ethanolic 2N-KOH (10 ml.) was added and the reaction was allowed to proceed for 30 min. After this time the mixture was diluted with water (200 ml.) and extracted with ethyl acetate (3 × 40 ml. portions); the organic phase was back-extracted with aq. saturated $NaHCO_3$ (3 × 10 ml. portions) and the combined aqueous portions were acidified with 2N-HCl and extracted further with ethyl acetate (3 × 30 ml. portions). The latter extract was washed with a little water, benzene was added and the solution was evaporated to dryness *in vacuo*; the resulting gum (3·72 g., 84%) was chromatographically pure, with R_F 0·81 in solvent B. A portion of this compound (0·7 g.) was dissolved in dry ether (15 ml.) and *cyclo*hexylamine (0·185 g., 1 mol.prop.) was added, leading to the immediate formation of *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*DL*-valine* cyclo*hexylamine* salt (0·68 g., 80%), m.p. 136–138°. Recrystallization from ethyl acetate–*cyclo*hexane gave analytically pure material, m.p. 139–140° (Found: C, 63·9; H, 7·4; N, 7·5. $C_{29}H_{41}O_5N_3S$ requires C, 64·1; H, 7·5; N, 7·5%).

The protected dipeptide (V*a*; 3·72 g.) was dissolved in liquid ammonia (100 ml.) in a three-necked 250 ml. flask fitted with a mechanical stirrer. Sodium was added in small portions, with stirring, until a permanent blue colour was obtained; 1 g. was required. Excess of sodium was destroyed with $(NH_4)_2SO_4$ and the liquid NH_3 was evaporated as rapidly as possible, last traces being removed by evacuating the flask at 40° for 2 hr. followed by 3 hr. in a desiccator over conc. H_2SO_4 at 37°. The residue was dissolved in 0·5N-H_2SO_4 (75 ml.) and a solution of mercuric sulphate (10% in 5% H_2SO_4) was added until precipitation was complete (12 ml.). After standing overnight at 0° the precipitate was washed with water, made into a fine powder by grinding and treated in aqueous suspension with H_2S to decompose the mercury salt. The mixture was filtered from mercury sulphide and the filtrate was evaporated to dryness *in vacuo* to yield 2·2 g. of a crude product which was desalted on a column of Zeo-Karb 215 (50 g.) (The Permutit Co. Ltd., London) by adsorption on to the H^+ form and elution with aq. 0·05N-NH_3 soln. to give crude L-cystinyl-DL-valine (VIII*a*; 1·6 g., 62%). Chromatography in solvent A revealed the presence of three components of R_F 0·19, 0·26 and 0·35 respectively, presumably representing the diastereoisomeric forms of the peptide. The peptide (1·3 g.) was suspended in water (20 ml.) and dissolved by the dropwise addition of dilute aq. NH_3 soln.; excess of NH_3 was removed in a desiccator and on addition of ethanol and ether 320 mg. of solid was obtained which ran as one spot, R_F 0·20, in solvent A. The product was dried for 6 hr. at 100° over P_2O_5. A sample (100 mg.) was hydrolysed in 6N-HCl for 64 hr. at 105° and evaporated to dryness *in vacuo*; the residue was dissolved in water and adjusted to pH 8 with aq. 0·15N-NH_3 soln. to precipitate the cystine liberated by hydrolysis. The latter was filtered off and the filtrate, after treatment with bromine–water to oxidize any remaining cystine to cysteic acid, was placed on a Zeo-Karb 215 (H^+ form) column and eluted with water until the eluate was neutral. Valine was displaced from the column with aq. 0·15N-NH_3 soln. and isolated. Optical rotations of both the cystine and valine were determined and compared with the rotations of cystine and valine isolated after treatment of synthetic equimolar mixtures under identical conditions of hydrolysis. The results, shown in Table 1, indicated that the peptide isolated by crystallization was *L-cystinyl-D-valine dihydrate* (VIII*b*), m.p. > 300°, $[\alpha]_D^{22}$ + 21·2° (*c*, 0·8 in 5N-HCl) (Found: C, 40·7; H, 7·2; N, 11·8. $C_{16}H_{30}O_6N_4S_2,2H_2O$ requires C, 40·5; H, 7·2; N, 11·8%). The R_F in solvent A was 0·20. Attempts to separate the other optical isomers from the mother liquors by ion-exchange chromatography on Zeo-Karb 225 were unsuccessful.

Table 1. *Effect of acid hydrolysis on the optical rotation of cystine and valine*

Cystine and valine were isolated from the peptide after hydrolysis with 6N-HCl at 105° for 64 hr. and from synthetic mixtures which had been submitted to the same hydrolytic procedure. The optical rotations refer to 1% solutions in 5N-HCl.

	$[\alpha]_D^{22}$	
	Cystine	Valine
Synthetic peptide	– 74°	– 27·4°
L-Cystine + DL-valine	– 57	0
L-Cystine + L-valine	– 60	+ 24·0
L-Cystine + D-valine	– 83	– 25·6

Optically specific syntheses

Benzyloxycarbonyl-*S*-benzyl-L-cysteine (II; 6 g.) and L-valine ethyl ester hydrochloride (Vaughan & Eichler, 1953) (3·15 g., 1 mol.prop.) were shaken together in dry ethylene dichloride (50 ml.) for 40 min. to give a clear solution. Triethylamine (1·76 g., 1 mol.prop.) and di*cyclo*hexylcarbodi-imide (3·86 g., 1 mol.prop.) (Sheehan & Hess, 1955) were added, with cooling; the mixture was shaken mechanically for 2½ hr. and allowed to stand at room temperature overnight. The insoluble di*cyclo*hexylurea formed during the reaction was filtered off, the solvent was evaporated *in vacuo* and the residue was dissolved in ethyl acetate (100 ml.). The latter solution was extracted successively with water (2 × 30 ml.), N-HCl (2 × 30 ml.), water (2 × 30 ml.), aq. 2% $NaHCO_3$ (2 × 30 ml.) and water (2 × 30 ml.); the organic phase was evaporated to dryness *in vacuo* in the presence of benzene to yield an oil which on subsequent evaporation with ethanol yielded a crystalline solid. After standing overnight under dry light petroleum (b.p. 100–120°) the solid was filtered off and dried *in vacuo* to give *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*L*-valine ethyl ester* (IV*c*; R = C_2H_5) (7·52 g., 92%), m.p. 78°, $[\alpha]_D^{24}$ – 38·0° (*c*, 1·0 in dioxan) (Found: C, 63·4; H, 6·8; N, 6·1%).

A similar preparation with L-*valine benzyl ester hydrobromide* (prepared by the method of Ben-Ishai & Berger, 1952), m.p. 118° (Found: C, 49·9; H, 6·6; N, 4·7. $C_{12}H_{18}O_2NBr$ requires C, 50·0; H, 6·3; N, 4·8%), gave *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*L*-valine benzyl ester* (IV*c*; R = $CH_2 \cdot C_6H_5$), m.p. 62–64°, $[\alpha]_D^{24}$ – 24·4° (*c*, 1·56 in benzene) (Found: C, 67·7; H, 6·3; N, 5·3%).

The ethyl ester (IV*c*; R = C_2H_5; 7·2 g.) was dissolved in acetone (105 ml.) and N-NaOH (75 ml., 5 mol.prop.) was added. The mixture was shaken mechanically at room temperature for 30 min., filtered and the filtrate was poured into water (850 ml.); the aqueous solution was extracted with ethyl acetate (3 × 200 ml.), the organic phase then being back-extracted with aq. 2% $NaHCO_3$ (3 × 100 ml.). The combined aqueous portions were acidified to Congo red with 2N-HCl in the usual way and the acidified solution was extracted thoroughly with ethyl acetate (3 × 200 ml.). After drying overnight ($MgSO_4$) the solvent was removed *in vacuo* to yield a gum (6·74 g.) which gave one spot, R_F 0·85 in solvent B. For analysis a small quantity of material was converted into the *cyclo*hexylamine salt in the manner described above. Several recrystallizations from ethyl acetate–*cyclo*hexane (containing 1 drop of *cyclo*hexylamine) gave *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*L*-valine cyclohexylamine salt*, m.p. 145–146° (Found: N, 7·53. $C_{29}H_{41}O_5N_3S$ requires N, 7·7%).

The saponified material (V*c*; 5·39 g.) was suspended in dry acetic acid (108 ml.) containing HBr (17·5 g.) and allowed to stand at room temperature for 45 min.; the resulting clear solution was freeze-dried to yield 5·1 g. of crude product (VI*c*), R_F 0·77 in solvent A, which was dissolved in water (50 ml.) and extracted with ether to remove benzyl bromide. The aqueous solution was then applied to a Zeo-Karb 215 (30 g.) column in the H^+ form; the column was eluted with water until the eluate was neutral and the product was finally displaced from the column with aq. 0·15N-NH_3 soln.; evaporation of the solution to dryness, followed by crystallization of the residue from ethanol–ether, gave 2·9 g. (77%) of *S-benzyl-*L*-cysteinyl-*L*-valine* (VI*c*), m.p. 198–200°, $[\alpha]_D^{22}$ +16·0° (*c*, 1·0 in ethanol) (Found: C, 58·2; H, 7·39; N, 9·05. $C_{15}H_{22}O_3N_2S$ requires C, 58·0; H, 7·1; N, 9·05%). The R_F values were 0·85 in solvent A and 0·53 in solvent B.

Liquid NH_3 was treated with sodium until a permanent blue colour was obtained and was then distilled in a closed system into a flask cooled in liquid air. When 50 ml. had been collected, the *S*-benzyl peptide (620 mg.) was added and sodium was introduced into the flask in small quantities, with mechanical stirring, until a permanent (7 min.) blue colour was obtained; a total of 154 mg. was required. Ammonium sulphate (450 mg.) was added and the liquid NH_3 was evaporated as quickly as possible, last traces being removed in a desiccator over conc. H_2SO_4. The residue was suspended in water, the pH adjusted to pH 8 with N-HCl, a trace of dil. aq. $FeCl_3$ was added and air was bubbled through the solution until the blue colour was discharged (15 min.). The peptide was absorbed on a Zeo-Karb 215 column (30 g., 2 mol.prop.), which was eluted with water to neutrality in the usual way and the peptide was then displaced from the column with aq. 0·15N-NH_3 soln. Evaporation of the eluate *in vacuo* gave 0·35 g. (79·5%) of crude product. Purification was effected by dissolving the product in the minimum of water containing a trace of dil. NH_3 soln. and then adding ethanol and ether; the product was dried at 100° for 6 hr. over P_2O_5 to give pure *L-cystinyl-*L*-valine monohydrate* (VIII*c*), m.p. > 300°, $[\alpha]_D^{27}$ −21·8° (*c*, 0·78 in 5N-HCl) (Found: C, 42·1; H, 6·91; N, 12·7. $C_{16}H_{30}O_6N_4S_2,H_2O$ requires C, 42·1; H, 7·04; N, 12·3%). The peptide gave one spot, R_F 0·30 in solvent A. The optical purity of this material was confirmed by hydrolysis, the isolated cystine having $[\alpha]_D^{24}$ −77·0 (*c*, 1·0 in 5N-HCl) and the valine $[\alpha]_D^{25}$ +24·0° (*c*, 1·0 in 5N-HCl).

Bis-benzyloxycarbonyl-L-cystine (5·1 g.) (Bergmann & Zervas, 1932) and D-valine ethyl ester hydrochloride (3·63 g.) were suspended in dry ethylene dichloride (40 ml.) and a clear solution was obtained after addition of dry ethyl acetate (20 ml.). Triethylamine (2·02 g.) and di-*cyclo*hexylcarbodi-imide (4·53 g., 1·1 mol.prop.) were added and the reaction was allowed to proceed in the usual way. The precipitated product was separated from di*cyclo*hexylurea by dilution of the reaction mixture with warm ethyl acetate (100 ml.), and after removal of the urea by filtration the solution was treated as described in previous experiments to give 6·98 g. (90%) of crude peptide. Recrystallization from ethyl acetate–ethanol–light petroleum (b.p. 80–100°) gave *bis-benzyloxycarbonyl-*L*-cystinyl-*D*-valine ethyl ester* (5·5 g.; 78%), m.p. 179–180°, $[\alpha]_D^{22}$ +6·0° (*c*, 0·5 in dioxan) (Found: C, 56·9; H, 6·6; N, 7·3. $C_{36}H_{50}O_{10}N_4S_2$ requires C, 56·7; H, 6·6; N, 7·3%). This material could not be saponified and therefore this route to the peptides via cystinyl intermediates was abandoned.

Benzyloxycarbonyl-*S*-benzyl-L-cysteine (11; 6 g.) and D-valine ethyl ester hydrochloride were coupled under conditions identical with those described for the fully protected L-peptide (IV*c*), to give *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*D*-valine ethyl ester* (IV*b*; R = C_2H_5), 7·63 g., (93%), m.p. 120°, $[\alpha]_D^{22}$ +19·0° (*c*, 1·0 in dioxan) (Found: C, 63·6; H, 6·5; N, 5·9%). The R_F in solvent B was 0·96.

The ester (IV*b*; 6·15 g.) was saponified for 40 min. in acetone (160 ml.) and N-NaOH (65 ml., 5 mol.prop.) and treated in the usual way to give *benzyloxycarbonyl-S-benzyl-*L*-cysteinyl-*D*-valine* (V*b*) (4·6 g., 80%), m.p. 112–114°, $[\alpha]_D^{24}$ −31° (*c*, 1·0 in ethanol) (Found: C, 62·2; H, 6·55; N, 6·5%. The R_F in solvent B was 0·85. $C_{23}H_{28}O_5N_2S$ requires C, 62·2; H, 6·3; N, 6·3%).

The above product (V*b*; 4·48 g.) was treated with a 2M solution of HBr in acetic acid at room temperature for 45 min.; the reaction mixture was treated as described for the L-isomer (VI*c*) to give fine needles of *S*-benzyl-L-cysteinyl-D-valine (VI*b*) (2·0 g., 64%), m.p. 207–209°, $[\alpha]_D^{23}$ +17·6° (*c*, 1·6 in 2·5N-HCl) (Found: C, 57·7; H, 7·2; N, 8·8. Calc. for $C_{15}H_{22}O_3N_2S$: C, 58·04; H, 7·1; N, 9·0%). Holley, Peel, Luz & Folkers (1952) found m.p. 204–209°, $[\alpha]_D^{23}$ +23·5° (*c*, 1·6 in 2·5N-HCl).

The *S*-benzyl peptide (VI*b*; 2·03 g.) was reduced with sodium in liquid NH_3 (720 mg. in 75 ml.) in the usual way and the product was obtained as described above for the L-isomer (VIII*c*); recrystallization gave cystinyl-D-valine dihydrate (800 mg., 56%), m.p. > 300°, $[\alpha]_D^{22}$ +29·6° (*c*, 1·01 in 5N-HCl) (Found: C, 40·0; H, 7·2; N, 11·6%). The product ran as one spot, R_F 0·19, on Whatman no. 4 paper in solvent A. Attempts to remove completely the water of crystallization by rigorous drying *in vacuo* at 100° were unsuccessful; a loss in weight of 7·5% was obtained (theory 9%). The optical configuration of the peptide was obtained by hydrolysis and subsequent isolation of the cystine and valine. The cystine gave $[\alpha]_D^{22}$ 0° (*c*, 1·0 in 5N-HCl), and the valine gave $[\alpha]_D^{22}$ −28·0° (*c*, 1·0 in 5N-HCl); the peptide was therefore DL-*cystinyl-*D*-valine* (VIII*d*).

Synthesis of ^{14}C*-labelled peptides*

Resolution of DL-[carboxy-^{14}C]*valine.* DL-[*carboxy-*^{14}C]-Valine (117·1 mg., 1 mc) was converted into the chloracetyl derivative (Birnbaum, Levintow, Kingsley & Greenstein, 1952), diluted with unlabelled chloracetyl-DL-valine (6·15 g.) and resolved with acylase I by the method of

Table 2. *Summary of synthesis of labelled peptides and intermediates*

The radiochemical purity has been calculated from the specific radioactivities of the original valine and products as follows:

$$\frac{\text{specific radioactivity of product} \times \text{mol.wt. of product}}{\text{specific radioactivity of valine} \times \text{mol.wt. of valine}} \times 100.$$

Optical rotations were determined under the same conditions as for the unlabelled materials (see text); —, signifies value not determined.

Compound	Yield (%)	M.p.	Specific activity (μc/g.)	Radiochemical purity (%)	$[\alpha]_D$
IV*b*	85	119–120°	47·4	93	−18·1°
IV*c*	84	75–77	48·6	94	−39·0
V*b*	84	111–113	55·1	103	−29·8
V*c*	90	110–111	57·6	105	—
VI*b*	74	206–209	70·0	92	—
VI*c*	61	199–201	74·0	97	—
VIII*b*	59·5	>300	104·8	96	+23·0
VIII*c*	75·5	>300	104·8	96	−21·5

Greenstein (1954). After the incubation was complete the solution was deproteinized by boiling, then filtered and concentrated *in vacuo*. The concentrated solution was loaded on to a Zeo-Karb 225 column (The Permutit Co. Ltd., London) (200 g., 60 cm. × 2 cm. diam.) and eluted with water until neutral; the acidic fractions were bulked, evaporated to dryness *in vacuo* and hydrolysed with 2·5 N-HCl (100 ml.) at 100° for 4 hr. After two evaporations to dryness *in vacuo* to remove excess of HCl, the residue was freeze-dried from water (20 ml.); the product was heated at 60° for 3 hr. in a vacuum-sublimation apparatus to remove chloracetic acid. The yield of D-[*carboxy*-^{14}C]valine hydrochloride was 2·54 g. (100%), $[\alpha]_D^{21}$ −26·0° (*c*, 1·0 in 5 N-HCl), specific activity 155·2 μc/g. L-[*carboxy*-^{14}C]Valine was displaced from the Zeo-Karb 225 column with 2·5 N-HCl and the eluate was fed into a fraction collector. Fractions containing valine (ascertained by paper chromatography in solvent A) were bulked and freeze-dried to give L-[*carboxy*-^{14}C]valine hydrochloride (2·45 g., 84%), $[\alpha]_D^{22}$ + 25·0° (*c*, 1·02 in 5 N-HCl), specific activity 159·2 μc/g.

Summary of synthesis of ^{14}C-*labelled peptides.* The route followed in the synthesis of L-cystinyl-L-[*carboxyl*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine was identical with that described in the section on optically specific syntheses as far as the stage of removal of the benzyloxycarbonyl group. The liquid-NH_3 reduction stage was carried out with metallic lithium instead of sodium in an attempt to simplify the removal of salt from the product; the reduction went smoothly but desalting was only partially successful in that after a first crop of salt-free peptide had been obtained by crystallization it was necessary to use a Zeo-Karb 215 column to complete the process. The details of yields, specific activity etc. are shown in Table 2. The L-cystinyl-L-[*carboxy*-^{14}C]valine was subsequently found to contain a small quantity of more acidic material which was removed as follows. The peptide (200 mg., 104·8 μc/g.) was dissolved in 0·5 M-acetic acid (5 ml.) and applied to a column of De-Acidite FF (The Permutit Co. Ltd., London) in the acetate form (25 g.). The column was eluted with 0·5 M-acetic acid at a rate of 5 ml./hr.; 5 ml. fractions were collected with a fraction collector. The yield of pure disulphide peptide was 180 mg. and was isolated as the diacetate, as indicated by the drop in specific activity to 89·2 μc/g. (diacetate requires 90 μc/g.). Another sample of peptide (104·8 μc/g.), which had not been subjected to treatment with De-Acidite FF, was dissolved in 0·5 M-acetic acid, freeze-dried and then dried to constant weight at 100° *in vacuo* over P_2O_5; the product had a specific activity of 86·7 μc/g., again in fair agreement with the required specific activity of the diacetate salt. Attempts to remove all the acetic acid by prolonged drying were unsuccessful; products with specific activities intermediate between those of the anhydrous and salt forms of the peptide were obtained.

SUMMARY

1. The synthesis of L-cystinyl-L-valine, L-cystinyl-D-valine and DL-cystinyl-D-valine by routes using the mixed-anhydride and dicyclohexylcarbodi-imide-coupling methods is described.

2. The problems of racemization during the removal of the protecting groups and removal of salt from the free peptides are discussed.

3. The synthesis of L-cystinyl-L-[*carboxy*-^{14}C]-valine and L-cystinyl-D-[*carboxy*-^{14}C]valine is reported.

The authors wish to express their appreciation of skilled technical assistance given by Mr E. J. Toms.

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The Utilization of L-Cystinyl-L-Valine for Penicillin Biosynthesis

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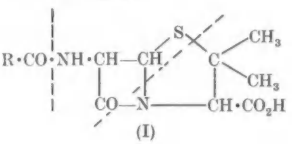
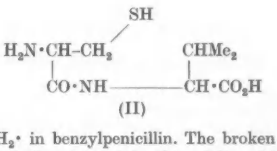
There is now good evidence that the thiazolidine–β-lactam ring structure of penicillin (I) is derived from L-cystine and L-valine (for recent reviews see Demain, 1959; Arnstein, 1957) and oxidative ring-closure of the dipeptide cysteinylvaline (II) or of a closely related derivative has been suggested as a possible mechanism for the biosynthesis (Arnstein & Crawhall, 1957; Birch & Smith, 1958). In order to investigate the metabolism of this dipeptide in relation to penicillin biosynthesis, the disulphide form of (II) was synthesized from ^{14}C-labelled valine (Arnstein & Morris, 1960), both L-cystinyl-L-[*carboxy*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine being prepared.



(I)



(II)

R=$C_6H_5\cdot CH_2\cdot$ in benzylpenicillin. The broken lines indicate the metabolic origin of the thiazolidine–β-lactam ring structure from cysteine and valine.

In this paper it will be shown that *Penicillium chrysogenum* utilizes L-cystinyl-L-valine for penicillin formation without obligatory cleavage of the peptide bond. In contrast, the peptide containing D-valine was not taken up by the mycelial cells and appeared to be metabolically inert. Most of this work has been described by Arnstein & Morris (1959).

EXPERIMENTAL

Organism and maintenance of cultures. The strain of *Penicillium chrysogenum* used was WIS 51–20 F 3, obtained from Dr W. J. Halliday, University of Queensland. Subcultures and spore inocula were prepared as described by Arnstein & Grant (1954).

Preparation of washed mycelium. Fermentations were carried out in the manner described by Halliday & Arnstein (1956) with the exception that the sterile potassium phenylacetate (0·1%/24 hr.) was added in two discrete portions after 24 and 40 hr. fermentations respectively. After 64–70 hr. the mycelium was harvested, washed three times by resuspension in 0·01 M-potassium phosphate buffer, pH 7, once by resuspension in 0·1 M-potassium phosphate buffer, pH 7, and filtered to give a mycelium of 13–15% dry wt.; the mycelium was then either used at once or stored at +1° under O_2 overnight for use on the following day. The potassium phosphate buffers referred to in this paper were prepared by diluting a M solution containing 136 g. of KH_2PO_4 and 40 g. of KOH/l.

Incubation of washed mycelium with precursors. Portions of mycelium (5 g. wet wt.) were added to duplicate 500 ml. conical flasks containing 50 ml. of ice-cold 0·01 M-potassium phosphate buffer, pH 7, 0·005% of potassium phenylacetate and 1% (w/v) of glucose (hereafter referred to as the medium) in the presence of a known quantity of ^{14}C-labelled precursor. After mixing at 0°, 1 ml. was removed from each flask, filtered and the filtrate was frozen and kept for uptake estimations (see below). The flasks were warmed rapidly to 25° and incubated on a rotary shaker (approx. 170 rev./min.) at this temperature for the required period. The flasks were then removed, chilled in ice and the medium was filtered by suction; 1 ml. of filtrate was removed from each flask for uptake estimation and frozen as before. The mycelial pads were immediately stored in solid CO_2 and the filtrate from each incubation was made up to 100 ml. in calibrated flasks with 0·1 M-potassium phosphate buffer, pH 7. After thorough mixing, portions (1 ml.) were

removed for bioassay and the solutions were made up to 100 ml. with 0·02 M-phosphate buffer, pH 7 (1 ml.), containing carrier sodium benzylpenicillin (50 mg.; potency 1670 i.u./mg.); after thorough equilibration of the contents the flasks were stored at 0° before isolation of the penicillin. In subsequent experiments conditions were altered slightly in that the incubation medium was warmed to 25° before adding the mycelium at the beginning of the experiment, and the filtrate at the end of the incubation was diluted to 50 ml., 75 mg. of carrier penicillin being added; the latter changes increased the efficiency of penicillin isolation.

Uptake estimations. The 1 ml. samples removed from each flask at the beginning and end of the incubation period were placed in a boiling-water bath for 2 min.; after cooling, a portion from each sample was placed on a 2 cm.² polythene disk (containing lens tissue). The disks were dried *in vacuo* and weighed and counted. The counts were corrected to 'infinite thinness', with a self-absorption curve prepared with samples of known radioactivity plated under identical conditions. The percentage uptake was then calculated from the initial and final activities in the medium.

Penicillin bioassay. Penicillin was estimated by the cup–plate method with *Bacillus subtilis* (ICI strain N.C.T.C. 8241) as test organism (Humphrey & Lightbown, 1952).

Isolation of penicillin. Penicillin was extracted from the filtrate at 0° by acidification to pH 2 with 25% (v/v) phosphoric acid and extraction with ether. The volume of the solution was decreased by successive partitioning between ether at pH 2·0 and phosphate buffer at pH 7·0 (Smith & Hockenhull, 1952). The penicillin was isolated as the *N*-ethylpiperidine salt, which was purified to constant radioactivity by recrystallization from chloroform–acetone (1:4, v/v).

Radioactivity measurements. Unless otherwise stated, samples were mounted on 1 cm.² polythene disks and counted at infinite thickness, as described by Arnstein & Clubb (1958).

Isolation of mycelial protein. The mycelium was defatted by boiling with ethanol and ether. The product was extracted with 5% (w/v) trichloroacetic acid (25 ml./g.) for 30 min. at room temperature, 15 min. at 90° and finally for 15 min. at room temperature. The residue was washed with ethanol and ether and dried *in vacuo* over P_2O_5. Samples were mounted on 1 cm.² disks and counted as described above.

Labelled compounds. L-Cystinyl-L-[*carboxy*-^{14}C]valine and L-cystinyl-D-[*carboxy*-^{14}C]valine were synthesized as described by Arnstein & Morris (1959, 1960). The former peptide was isolated as the acetate salt. Uniformly ^{14}C-labelled L-valine, hereafter described as L-[U-^{14}C]valine, was obtained from The Radiochemical Centre, Amersham, Bucks.

Assessment of results. In order to facilitate assessment of the results, all specific activities have been corrected for differences of uptake and initial specific activities of precursors to give figures corresponding to 1 μc being available in the cells.

RESULTS

Incubation of the dipeptides with washed mycelium (Expt. 1)

Duplicate flasks containing L-cystinyl-L-[*carboxy*-^{14}C]valine acetate (0·49 μc, 5·9 mg.; equivalent to 0·0171 m-mole of valine), L-cystinyl-D-[*carboxy*-^{14}C]valine (0·49 μc, 4·7 mg.; equivalent to 0·0171 m-mole of valine) and L-[U-^{14}C]valine (0·215 μc, 2·5 mg.; 0·0171 m-mole) respectively were incubated with washed mycelium, in the medium described above, for 1, 2 and 4 hr. respectively. Uptake of the labelled precursors and incorporation of radioactivity into both penicillin and mycelial protein were determined. Uptake, expressed as the percentage of total activity available, is shown in Fig. 1. Good agreement (usually better than 2%) between duplicates was obtained and mean values have been plotted. At 1 hr. 69% of the added L-peptide had been taken up, but only 36% of the valine. After 2 hr. the uptake of L-peptide was virtually complete but that of valine rose to a similar figure only after a further 2 hr. No measurable uptake of D-peptide occurred and there was no radioactivity in either penicillin or mycelial protein in the experiments with this peptide.

Penicillin yields are shown in Table 1. Considering the incubations containing D-peptide as a control, penicillin production from L-peptide appeared to be very slightly inhibited in the early stages but was stimulated to some extent after 2 hr.; the reverse effect, only to a smaller extent, was observed in penicillin production from valine.

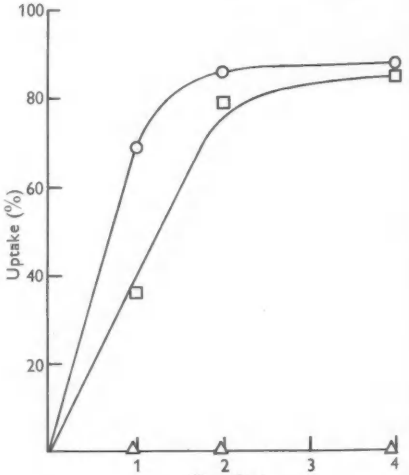


Fig. 1. Uptake of L-cystinyl-L-[*carboxy*-^{14}C]valine (○), L-cystinyl-D-[*carboxy*-^{14}C]valine (△) and L-[U-^{14}C]valine (□) into washed mycelium of *P. chrysogenum* WIS 51–20 F3 at 24° (see text, Expt. 1). Each point represents the mean percentage uptake of duplicate experiments.

The mean specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein derived from incubations in the presence of L-peptide or L-valine for 1, 2 and 4 hr. respectively are shown in Fig. 2. At 1 hr. the peptide was more efficiently incorporated into penicillin than was valine; at 2 hr. the efficiencies were almost equal and at 4 hr. valine was incorporated more efficiently. At all times the specific activity of mycelial protein derived from peptide was considerably less than that from valine. The ratio of penicillin to protein activity was much higher at all times with peptide as precursor than it was with valine, as shown in Table 2.

Table 1. *Penicillin production by washed mycelium in the presence of* ^{14}C*-labelled valine and* ^{14}C*-labelled cystinylvaline*

In Expt. 1 (see text), washed mycelium of *P. chrysogenum* WIS 51–20 F3 was suspended in medium containing ^{14}C-labelled L-peptide acetate, D-peptide or L-[U-^{14}C]-valine. In Expt. 2, the experimental conditions were the same except that the D-peptide was not used and GSH (66 mg., 10 mol.prop.) was present. In Expt. 3, L-[U-^{14}C]-valine was incubated with and without GSH (66 mg., 10 mol.prop.) under the same conditions. Results are the means of duplicate flasks.

		Time (hr.)		
		1	2	4
		Penicillin production (i.u./flask)		
Expt.	Precursor			
1	L-Peptide	201	537	1260
	D-Peptide	226	491	976
	L-Valine	242	466	847
2	L-Peptide	192	338	—
	L-Valine	200	444	—
3	L-Valine (+GSH)	977	—	—
	L-Valine (control)	910	—	—

Table 2. *Comparison of the ratio of specific activities of penicillin and protein derived from* ^{14}C*-labelled* L*-cystinylvaline and* L*-*[U-^{14}C]*valine respectively*

For details of experiments see text.

		Ratio of specific radioactivities: penicillin/protein	
Expt.	Incubation time (hr.)	L-Peptide as precursor	L-Valine as precursor
1	1	248	94
	2	96	55
	4	54	31
2	1	268*	104*
	2	136*	41·5*
3	1	—	146*
	1	—	109

* With glutathione in the medium.

Reduction of L*-peptide with glutathione*

It seemed possible that the peptide might be utilized more efficiently in the sulphydryl form, and reduction of the disulphide with excess of glutathione (GSH) was investigated as this appeared to be a convenient method. L-Cystinyl-L-[*carboxy*-^{14}C]valine acetate (5·56 mg., 88·8 μC/g.) was dissolved in 0·1 M-phosphate buffer, pH 7 (5 ml.), and diluted to 25 ml. with water. Glutathione (66 mg.; 10 mol.prop.) was dissolved in water (25 ml.) and the two solutions were mixed for 4 hr.; to each portion was added *N*-ethylmaleimide (0·4 ml., 10 mg./ml.) and the samples were freeze-dried. Chromatography on Whatman paper no. 1 in butanol–acetic acid–water (63:10:27, by vol.), followed by scanning for radioactivity on an automatic paper-chromatogram scanner, revealed that very rapid reduction of the peptide to the thiol form had occurred; no radioactivity was found at the R_F of cystinylvaline, a small amount was found at the origin and over 80 % occurred at the R_F of authentic cysteinylvaline *N*-ethylmaleimide.

Incubation of precursors in the presence of glutathione (Expt. 2)

Duplicate flasks containing L-cystinyl-L-[*carboxy*-^{14}C]valine acetate (0·49 μC, 5·9 mg.; equivalent to 0·0171 m-mole of valine) or L-[U-^{14}C]valine (1·063 μC, 2·5 mg.; 0·0171 m-mole) were incubated, as described above, with washed mycelium for 1 and 2 hr. periods in the presence of GSH (66 mg., 10 mol.prop.). Bioassay figures are shown in Table 1.

The uptake of peptide was unaffected by the presence of GSH, the percentage uptakes at 1 and 2 hr. being 68·5 and 80 % respectively, compared with 68·8 and 85·8 % in Expt. 1 for corresponding

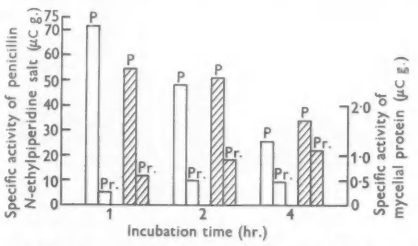


Fig. 2. Specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein isolated after incubation of washed mycelium of *P. chrysogenum* WIS 51–20 F3 with L-cystinyl-L-[*carboxy*-^{14}C]valine acetate and L-[U-^{14}C]-valine respectively as described in the text (Expt. 1). Each value in the figure represents the mean of duplicate experiments. P, Penicillin; Pr., mycelial protein; unshaded and shaded areas refer to results from peptide and valine precursors respectively.

times; uptake of valine, however, was markedly decreased from 35·5 to 19·2 % and from 78·8 to 51·6 % respectively at the same times.

The specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein are shown in the left-hand portion of Fig. 3. Compared with incubation without GSH (Expt. 1, Fig. 2), incorporation of peptide into penicillin was only slightly increased at 1 hr. but the increase was greater at 2 hr. Incorporation of valine, however, was practically doubled at 1 hr. but was relatively unaffected at 2 hr. Incorporation into protein with peptide as precursor was only slightly affected by GSH but a more significant increase occurred with valine as precursor. The ratios of specific activity of penicillin and mycelial protein are shown in Table 2. Once again the ratios with peptide as precursor were higher than with valine.

Incubation of L-[U-^{14}C]*valine with and without glutathione* (*Expt.* 3)

Duplicate flasks containing L-[U-^{14}C]valine (2·5 mg., 425 μc/g.) were incubated with washed mycelium for 1 hr. in the presence of GSH (66 mg., 10 mol.prop.); similar flasks without GSH were used as controls.

Bioassay figures are shown in Table 1. The overall penicillin yields were lower from this batch of washed mycelium but there was no significant effect of GSH on penicillin production.

Uptake of valine was decreased by GSH from 47·4 to 33·1 %. The specific activities of penicillin and mycelial protein isolated are shown in the right-hand portion of Fig. 3. The increase in the specific activity of penicillin in the presence of GSH, observed in Expt. 2 (left-hand side of Fig. 3), was confirmed, although it was quantitatively not as great. Incorporation into mycelial protein was unaffected by GSH.

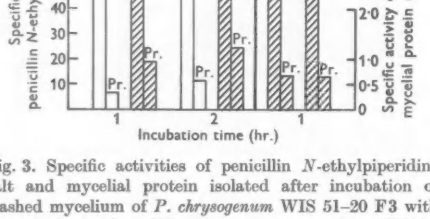


Fig. 3. Specific activities of penicillin *N*-ethylpiperidine salt and mycelial protein isolated after incubation of washed mycelium of *P. chrysogenum* WIS 51–20 F3 with L-cystinyl-L-[*carboxy*-^{14}C]valine acetate and L-[U-^{14}C]-valine in the presence of glutathione (Expt. 2); 10 equivalents of GSH with respect to peptide were used. The effect of glutathione on the incorporation of L-[U-^{14}C]valine (Expt. 3) is shown on the right-hand side. Conditions were as described in Fig. 2. P, Penicillin; Pr., mycelial protein; unshaded and shaded areas refer to results from peptide and valine precursors respectively.

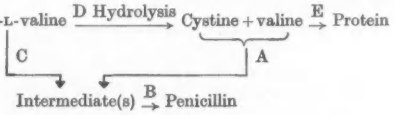
DISCUSSION

In the present work it was found that L-cystinyl-D-valine is not metabolized by suspensions of washed mycelium of *P. chrysogenum* to any significant extent. Since this result may be due to lack of uptake of this compound by the mycelial cells, no conclusion is possible about the possible function of this peptide in penicillin biosynthesis.

On the other hand, L-cystinyl-L-valine was taken up by the mycelium even faster than L-valine and it appeared to be readily metabolized. Thus on the basis of the reasonable assumption that cystinyl-valine sequences in the mycelial protein would occur only rarely, the incorporation of radioactivity from the labelled peptide into protein indicates considerable hydrolysis into cystine and valine, although the finding that the specific radioactivity of the protein was considerably lower than in comparable experiments with labelled valine (Fig. 2) suggests that hydrolysis was not complete.

Compared with valine, the peptide is a better precursor of penicillin than of protein, as shown most clearly by the comparison of the ratios of the specific radioactivities of penicillin and protein (Table 2). It would appear therefore that despite extensive hydrolysis some of the peptide was converted into penicillin by a pathway not involving breakdown into its constituent amino acids, provided that free valine and valine released by hydrolysis of the peptide are equally available for penicillin and protein synthesis. The possible importance of such a pathway may be roughly assessed on the basis of the following considerations. Since the maximum rate of penicillin formation (Table 1) is approx. 300 units (0·5 μmole/flask/hr.) and the uptake of peptide in the first hour (Fig. 1) may be calculated to be about 12 μmoles, the labelled peptide is likely to be present in considerable excess compared with unlabelled peptide synthesized endogenously. Any extensive conversion of the peptide into penicillin would therefore be expected to give rise to a product having the same molar radioactivity as the labelled peptide. A comparison of the highest specific radioactivity of penicillin derived from peptide (72 μc/g. of *N*-ethylpiperidine salt, 32 μc/m-mole; see Fig. 2) with that of peptide available in the cell (83 μc/m-mole with respect to valine) shows that there was in fact dilution of the specific

radioactivity. The direct conversion of L-cystinyl-L-valine into penicillin is thus relatively inefficient. It was thought that conversion of the peptide into the sulphydryl form might be rate-limiting, but reduction to L-cysteinyl-L-valine with excess of GSH did not result in any marked increase in the specific radioactivity of the penicillin, although GSH stimulated the utilization of valine to some extent.



Scheme 1

These observations are summarized in Scheme 1. In this scheme, reactions D and E would lead to labelling of protein from cystinylvaline, whereas reaction C would account for the preferential incorporation of dipeptide into penicillin relative to protein, compared with that observed with valine as precursor. Penicillin biosynthesis would, however, take place mainly by pathways (A and B) involving intermediates other than L-cystinyl-L-valine or L-cysteinyl-L-valine. In this connexion it may be significant that the intracellular sulphur-containing amino acid and peptide fraction of the mycelium contains substantial amounts of δ-(α-aminoadipyl)cystinylvaline whereas cystinylvaline could not be detected (Arnstein, Morris & Toms, 1959). Full discussion of the possible significance of this tripeptide in penicillin biosynthesis (cf. Arnstein *et al.* 1959) will be deferred, but it may be noted that stepwise biosynthesis of this peptide starting from the *N*-terminal amino acid, as with GSH, would not require the formation of cystinyl-valine as an intermediate.

SUMMARY

1. The incorporation of radioactivity from L-cystinyl-L-[*carboxy*-^{14}C]valine, L-cystinyl-D-[*carboxy*-^{14}C]valine and uniformly labelled L-[^{14}C]-valine into penicillin by washed mycelium of *Penicillium chrysogenum* WIS 51–20 F3 has been compared with their utilization for protein synthesis.

2. L-Cystinyl-D-[*carboxy*-^{14}C]valine was not metabolized to any significant extent.

3. L-Cystinyl-L-[*carboxy*-^{14}C]valine was used preferentially for penicillin biosynthesis, indicating that this peptide can be utilized without hydrolysis into cystine and valine.

4. The extent of this direct utilization of L-cystinyl-L-valine for penicillin formation appears to be quantitatively limited and the existence of a pathway of penicillin biosynthesis from cystine and valine, which does not involve cystinyl- or cysteinyl-valine as obligatory intermediates, is suggested.

We wish to thank Mr E. J. Toms for skilled technical assistance.

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The Behaviour of Pyridine Nucleotides of Mitochondria in a 'Saline Medium'

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The considerable volume of information obtained about the metabolism of the endogenous pyridine nucleotides of mitochondria has been derived from experiments in many different fields of research. Rarely have the investigations been concerned primarily with the reactions of the individual nucleotides themselves, though the work of Chance and his collaborators has thrown much light on the general pattern of behaviour of the mitochondrial coenzymes (for references to the need for investigations supplementary to this work see Chance & Baltscheffsky, 1958). Indeed, no systematic study of the reactions of the endogenous oxidized and reduced diphosphopyridine nucleotides and the oxidized and reduced triphosphopyridine nucleotides of mitochondria isolated by a standard procedure and subsequently incubated under a variety of experimental conditions has been reported. This paper describes an investigation of the reactivity of the pyridine nucleotides of mitochondria suspended in a 'saline medium' and the influence of the components of this medium on the behaviour of the coenzymes.

MATERIALS

5-*Ethyl*-5-iso*amylbarbiturate* (*amytal*). This was obtained from Eli Lilly and Co. Ltd., Basingstoke, and freshly prepared solutions of the sodium salt were used in each experiment.

2:4-*Dinitrophenol*. This was obtained from British Drug Houses Ltd., and stock solutions of this compound and of the metabolites used were prepared in 'saline medium' (see below) and stored at $-15°$ before use.

Adenosine triphosphate. This was obtained as commercial products from L. Light and Co. Ltd., Colnbrook, Bucks, and Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany.

All the other compounds used were commercial products. Reagents used in the analysis of pyridine nucleotides were as described by Bassham, Birt, Hems & Loening (1959).

METHODS

Preparation of media for the isolation and incubation of mitochondria. In experiments on the influence of nicotinamide on pyridine nucleotide metabolism, the basic medium was that of Chappell & Perry (1954), containing KCl (0·1M), 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4 (0·05M), ethylenediaminetetraacetic acid (EDTA; 1 mM), $MgSO_4$ (5 mM) and adenosine triphosphate (ATP; 1 mM). The sucrose and 'saline' media were prepared as described by Birt & Bartley (1960*a*); the latter medium was that of Chappell & Perry (1954), to which nicotinamide (25 mM) had been added.

Preparation of mince. Rat-liver mince was prepared as described by Bassham *et al.* (1959) except that the final suspension was made in 'saline medium'.

Preparation of mitochondria. (*a*) Rat-liver mitochondria. These were prepared by the method described by Birt & Bartley (1960*a*), except that the removal of the 'fluffy layer' was less complete and there was more variation in the concentration of mitochondria in the final suspension.

(*b*) Rat-brain mitochondria. These were prepared by the method of Bellamy (1959) except that nicotinamide (25 mM) was added to the sucrose medium. The isolated particles were washed twice and finally suspended at 0° in the 'saline medium'.

(*c*) Fly-muscle mitochondria. These were prepared by the method of Sacktor (1953), except that nicotinamide (25 mM) was added to the isolation medium (sucrose, 0·25M, and bovine albumin, 1%). The isolated particles were washed twice and finally suspended at 0° in 'saline medium'.

Incubations. These were carried out in open conical flasks which were agitated gently during incubation at the appropriate temperature in a Dubnoff metabolic shaking incubator (manufactured by Precision Scientific Co., Chicago, Ill., U.S.A.).

Measurement of the distribution of the pyridine nucleotides between the mitochondria and the suspending medium. The mitochondria in a sample of the suspension were separated from the supernatant fluid by centrifuging at 0° for 5 min. at 26 000 *g*. Extractions of the whole suspension and the supernatant were made and the coenzyme content of both was determined. The intramitochondrial pyridine nucleotide was determined by difference.

Measurement of uptake of oxygen. Uptake of O_2 was measured in the Warburg apparatus. The values given in the tables are rates of O_2 consumption derived from the total uptake of O_2 at the times shown in the tables.

Measurements of pyridine nucleotides. Oxidized diphosphopyridine nucleotide (DPN^+), reduced diphosphopyridine nucleotide (DPNH), oxidized triphosphopyridine nucleotide (TPN^+) and reduced triphosphopyridine nucleotide (TPNH) and total levels of oxidized and reduced pyridine nucleotides were measured by the fluorimetric

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method of Bassham *et al.* (1959). All values for amounts of coenzyme are given to two significant figures or to the nearest 0·05 μm-mole/mg. dry wt.

Measurement of protein content of mitochondria. The dried mitochondrial pellets from which the dry matter of the mitochondrial suspensions was determined were wet-ashed and the N content of suitable dilutions measured by direct nesslerization. The mitochondrial protein was assumed to contain 16% of N.

RESULTS

Relative stability of the pyridine nucleotides of rat-liver mince and mitochondria. A portion of a rat-liver mince was suspended in 'saline medium' (1 g. fresh wt./10 ml. of suspension) and the suspension was sampled and kept at 0° while mitochondria were isolated from the rest of the preparation. Further samples of both mince and the isolated mitochondria (at 0°) were taken, the temperature of the two preparations was raised to 25° and incubation continued for 1 hr. before the final sampling. The estimations showed that, whereas there was no loss of pyridine nucleotide in the mince kept at 0°, there was a slight increase in the proportion of the total coenzymes in the reduced form. During the incubation for 1 hr. at 25°, the rate of loss of the coenzymes was five times as great in the mince as in the mitochondrial suspension; the loss of reduced nucleotide was particularly great. Assuming that the mitochondria comprise 20% of the liver fresh weight and that the rate of loss of coenzymes was the same for the mitochondria in the two suspensions (both in the same medium), then the percentages of the total pyridine nucleotide present in the mitochondria at the beginning and end of the incubation were 34 and 89% respectively.

Influence of components of the isolation and incubation media on the pyridine nucleotides of mitochondria

Nicotinamide. Nicotinamide at 25 mM concentration in the sucrose medium used for isolation of mitochondria had little effect on the original levels of coenzymes in the isolated particles (Table 1). Though nothing is known about the extent to which the nicotinamide in the sucrose medium penetrates the mitochondria, it was found that the particles retained sufficient of it, even after the

Table 1. *Effect of nicotinamide on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Temperature of incubation, 25°; concn. of nicotinamide in both sucrose and Chappell–Perry media, 25 mM.

In this and subsequent tables, PN^+ represents $DPN^+ + TPN^+$, PNH represents DPNH + TPNH and PN represents $PN^+ + PNH$. For the meaning of the terms 'Absolute loss' (Abs. loss) and 'Relative loss' (Rel. loss) in this and subsequent tables, see text.

Rates of O_2 uptake (μl. of O_2/mg. dry wt./hr.) at (i) end of 15 min., (ii) end of 60 min.:

	(i)	(ii)		(i)	(ii)
(*a*)	6·4	3·7	(*c*)	4·4	0·9
(*b*)	2·8	0·9	(*d*)	2·8	0·9

Mitochondria prepared in sucrose medium

	(*a*) Incubated in Chappell–Perry medium						(*b*) Incubated in Chappell–Perry medium plus nicotinamide ('saline medium')					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Abs. loss (%)	Rel. loss (%)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Abs. loss (%)	Rel. loss (%)
0	0·70	1·70	2·40	2·4	27	—	1·75	1·55	3·30	0·90	0	—
15	0·75	1·25	2·00	1·7	39	17	2·40	0·85	3·25	0·35	2	2
60	0·40	0·75	1·15	1·9	65	52	2·10	0·30	2·40	0·14	27	27

Mitochondria prepared in sucrose–nicotinamide medium

	(*c*) Incubated in Chappell–Perry medium						(*d*) Incubated in Chappell–Perry medium plus nicotinamide ('saline medium')					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Abs. loss (%)	Rel. loss (%)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Abs. loss (%)	Rel. loss (%)
0	0·60	1·70	2·30	2·8	30	—	1·40	1·75	3·15	1·3	4	—
15	0·73	1·30	2·03	1·8	39	12	2·10	0·75	2·85	0·36	14	10
60	0·52	1·05	1·57	2·0	52	32	2·10	0·17	2·27	0·08	31	28

washings and incubation in a medium containing no nicotinamide, for its characteristic effects to be seen [compare the stability of coenzymes and the respiration rates in (*a*) and (*c*), Table 1]. Accordingly, it was retained at the same concentration in the sucrose as in the ionic medium used for incubation.

The addition of nicotinamide (25 mM) to Chappell–Perry medium used for final suspension of the particles in these experiments increased the initial amounts of pyridine nucleotides found in the isolated mitochondria (Table 1), even though all the washings and resuspensions (time approx. 45 min.) in this medium before the first extractions of coenzymes were done at 0°. The values for 'absolute loss' in Table 1 (and subsequent tables) are based on the decrease below the highest initial total found in each experiment [e.g., in Table 1, 3·30 μm-moles/mg. dry wt., in (*b*)]. The 'relative loss' (i.e. the percentage disappearance of the nucleotides in each individual mitochondrial suspension) was almost the same whenever the incubating medium contained nicotinamide, and only slightly greater when nicotinamide was present in the sucrose isolation medium alone (i.e. when it was absent from the incubation medium). When nicotinamide was omitted from both media, the loss during incubation was considerably greater.

Two other effects of nicotinamide were observed. First, it decreased the ability of mitochondria to maintain the nucleotides in the reduced state. This is shown in Table 1 [compare (*a*) and (*b*) and (*c*) and (*d*)]. Whereas the initial level of reduced coenzymes was similar in all four preparations, the final level in the presence of nicotinamide was relatively very small, so that the ratio of reduced coenzyme/oxidized coenzyme was greatly diminished. This diminution was enhanced by the protection of the oxidized coenzymes by the nicotinamide. Secondly, nicotinamide, whether present in the sucrose medium alone, in the 'saline medium' alone or in both, decreased the initial and final rates of endogenous respiration.

The influence of nicotinamide on mitochondria incubated with oxidizable substrate was tested in experiments with succinate and pyruvate (Tables 2 and 3).

Succinate. The stability of the coenzymes was affected in the same way by nicotinamide whether or not succinate was present, i.e. initial values were higher and the loss during incubation decreased. Values given in (*b*) and (*d*) of Table 2 illustrate the preferential protection of oxidized coenzymes. Succinate alone had little or no protective action: compare (*a*) and (*b*). At all times during incubation, the diminution produced by nicotinamide in

Table 2. *Effect of nicotinamide and succinate on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Temperature of incubation, 25°; concn. of succinate, 10 mM; concn. of nicotinamide, 25 mM. See also protocol to Table 1.

Rates of O_2 uptake (μl. of O_2/mg. dry wt./hr.) at (i) end of 15 min., (ii) end of 60 min.:

	(i)	(ii)		(i)	(ii)
(*a*)	3·6	1·8	(*c*)	2·4	1·2
(*b*)	21·6	2·8	(*d*)	22·8	11·6

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in Chappell–Perry medium

	(*a*) No substrate						(*b*) With succinate					
Time (min.)	PN+	PNH	PN	PNH / PN+	Abs. loss (%)	Rel. loss (%)	PN+	PNH	PN	PNH / PN+	Abs. loss (%)	Rel. loss (%)
0	0·4	2·9	3·30	7·3	19	—	0·29	2·8	3·09	9·7	25	—
15	0·85	2·1	2·95	2·5	25	11	0·18	3·0	3·18	17·0	22	0
60	0·60	1·4	2·00	2·3	51	39	0·31	1·8	2·11	5·8	49	31

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in Chappell–Perry medium plus nicotinamide ('saline medium')

	(*c*) No substrate						(*d*) With succinate					
Time (min.)	PN+	PNH	PN	PNH / PN+	Abs. loss (%)	Rel. loss (%)	PN+	PNH	PN	PNH / PN+	Abs. loss (%)	Rel. loss (%)
0	1·4	2·7	4·1	1·9	0	—	1·3	2·8	4·1	2·2	0	
15	2·6	0·83	3·43	0·32	16	16	0·9	3·5	4·4	3·9	0	0
60	2·7	0·45	3·15	0·17	23	23	1·4	2·4	3·8	1·7	7	7

the ratios of reduced to oxidized coenzymes, demonstrated again in the absence of substrate, was found also with succinate; though with this substrate, the greater part of the effect was due to the preservation of the oxidized coenzymes by the nicotinamide, rather than to a lowering of the level of reduced coenzymes [compare 15 min. values in (*b*) and (*d*), Table 2]. The ratios produced by succinate during the incubation declined more rapidly in the absence of nicotinamide.

The effect of nicotinamide on the respiration with succinate was markedly different from that on endogenous respiration. The rate with the added substrate was virtually unaltered by nicotinamide at the beginning of incubation and was maintained at a higher level (fourfold) after 1 hr. (Table 2). In this experiment the differences in the final respiration rates with succinate [(*b*) and (*d*)] were inversely proportional to the differences in the overall losses of coenzymes; the rate with nicotinamide was four times as great, and the loss of coenzymes four times less.

Pyruvate. Similar results were obtained with nicotinamide and pyruvate, which is much less effective than succinate in reducing pyridine nucleotides (Table 3). Nicotinamide stabilized the coenzymes and there was no decrease in the amounts of reduced nucleotide produced by the pyruvate. The preferential destruction of oxidized pyridine nucleotides in the absence of nicotinamide (compare 60 min. values, Table 3) caused the ratio of reduced coenzyme/oxidized coenzyme to increase throughout the hour of incubation.

Table 3. *Effect of nicotinamide and pyruvate on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. and for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temperature of incubation, 25°; concn. of nicotinamide 25 mM; concn. of pyruvate, 10 mM. See also protocol to Table 1.

	Mitochondria prepared in sucrose–nicotinamide medium											
	Incubated in Chappell–Perry medium plus pyruvate (10 mM)						Incubated in Chappell–Perry medium with nicotinamide ('saline medium') plus pyruvate (10 mM)					
Time (min.)	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2
0	1·15	3·00	4·15	2·6	—	—	1·75	3·05	4·80	1·7	—	—
15	1·05	3·45	4·50	3·3	0	13·2	0·96	3·90	4·86	4·1	0	10·4
60	0·48	2·70	3·18	5·6	23	5·6	1·20	3·15	4·35	2·6	8	5·6

Table 4. *Effect of ethylenediaminetetra-acetic acid on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt., and for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temperature of incubation, 25°; concn. of EDTA (sodium salt), 1 mM; concn. of pyruvate, 10 mM. Incubation medium without EDTA: KCl, 0·1 M; tris–HCl buffer (pH 7·4) 0·05 M; $MgSO_4$, 5 mM; ATP, 1 mM; nicotinamide 25 mM. See also protocol to Table 1.

	Mitochondria prepared in sucrose–nicotinamide medium. Incubated in medium without EDTA											
	(*a*) No substrate						(*b*) With pyruvate					
Time (min.)	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2
0	1·90	3·70	5·60	1·95	—	—	2·10	3·70	5·80	1·75	—	—
15	2·70	2·60	5·30	0·95	5	4·3	2·10	3·60	5·70	1·70	2	8·4
45	2·70	2·80	5·50	1·05	2	3·4	2·30	3·50	5·80	1·50	0	5·7
	Mitochondria prepared in sucrose–nicotinamide medium. Incubated in 'saline medium'											
	(*c*) No substrate						(*d*) With pyruvate					
Time (min.)	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2	PN+	PNH	PN	PNH / PN+	Loss (%)	Uptake of O_2
0	2·10	3·50	5·60	1·65	—	—	2·30	3·40	5·70	1·50	—	—
15	2·80	2·10	4·90	0·75	13	3·9	2·70	2·40	5·10	0·90	14	5·4
45	2·80	2·10	4·90	0·75	13	3·4	2·20	2·70	4·90	1·25	17	5·3

Adenosine triphosphate. Without added substrate, the omission of ATP had little effect on either the oxidized or reduced coenzyme, the only apparent result being a slight oxidation of the reduced nucleotides. With succinate, no differences were detected when ATP was omitted. However, the initial respiration, both with and without succinate, was increased by ATP. In the absence of ATP and without added substrate, the rate of oxygen uptake increased greatly during the incubation, and the level of reduced pyridine nucleotides fell to a very low value. Further reference to changes of this kind will be made later (see Birt & Bartley, 1960*b*).

Table 5. *Effect of magnesium on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt., and for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temperature of incubation, 25°; concn. of magnesium, 5 mM; concn. of pyruvate, 10 mM.

Incubation medium without Mg^{2+} ions: KCl, 0·1 M; tris–HCl buffer (pH 7·4) 0·05 M; EDTA, 1 mM; ATP, 1 mM; nicotinamide, 25 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in 'saline medium'

	(*a*) No substrate						(*b*) With pyruvate					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	1·40	2·60	4·00	1·9	—	—	1·35	2·80	4·15	2·1	—	—
15	1·90	1·80	3·70	0·9	8	3·0	0·55	3·45	4·00	6·3	4	9·0
60	3·15	0·30	3·45	0·1	14	1·1	1·15	2·60	3·75	2·3	10	7·0

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in medium without Mg^{2+} ions

	(*c*) No substrate						(*d*) With pyruvate					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	1·35	2·55	3·90	1·9	—	—	1·20	2·75	3·95	2·3	—	—
15	1·20	2·80	4·00	2·3	0	4·2	1·20	2·80	4·00	2·3	0	6·7
60	1·15	2·35	3·50	2·1	10	2·7	1·15	2·80	3·95	2·4	0	7·1

Table 6. *Effect of magnesium on the metabolism of mitochondrial pyridine nucleotides*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt., and for O_2 uptake as μl. of O_2/mg. dry wt./hr. Temperature of incubation, 25°; concn. of magnesium, 5 mM; concn. of succinate, 10 mM.

Incubation medium without Mg^{2+} ions: KCl, 0·1 M; tris–HCl buffer (pH 7·4), 0·05 M; EDTA, 1 mM; ATP, 1 mM; nicotinamide, 25 mM. See also protocol to Table 1.

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in 'saline medium'

	(*a*) No substrate						(*b*) With succinate					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	1·15	1·70	2·85	1·5	—	—	0·62	2·25	2·87	3·6	—	—
15	1·60	1·20	2·80	0·75	1	2·9	0·45	2·00	2·45	4·5	16	15·0
60	1·70	0·24	1·94	0·14	33	0·8	1·20	1·45	2·65	1·2	7	5·3

Mitochondria prepared in sucrose–nicotinamide medium. Incubated in medium without Mg^{2+} ions

	(*c*) No substrate						(*d*) With succinate					
Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2	PN^+	PNH	PN	$\frac{PNH}{PN^+}$	Loss (%)	Uptake of O_2
0	1·05	1·70	2·75	1·6	—	—	0·74	2·00	2·74	2·7	—	—
15	1·05	1·40	2·45	1·3	11	4·3	0·65	1·80	2·45	2·8	12	14·8
60	1·15	1·20	2·35	1·0	14	3·3	1·20	1·40	2·60	1·2	6	5·5

Ethylenediaminetetra-acetic acid. The initial levels of total pyridine nucleotide in isolated mitochondria were unaffected by the addition of EDTA, but the proportion of reduced coenzyme was decreased (Table 4). This effect of EDTA in lowering the ratio of reduced coenzyme/oxidized coenzyme became more apparent after incubation for 15 min., especially in the presence of an added substrate (e.g. pyruvate). The stability of the nucleotides was somewhat greater in the absence of EDTA, perhaps because of the greater proportion of reduced coenzymes and the lower rate of leakage from the particles (see Table 11). The initial rates of oxygen uptake, both with and without added substrate, were decreased by EDTA, but the subsequent decline in the respiration rate was not as great as when EDTA was omitted.

Magnesium. The initial amounts and proportions of the coenzymes in isolated mitochondria were unaffected by added magnesium (Tables 5 and 6). However, it promoted the oxidation of reduced pyridine nucleotide during incubation without added substrate, even though the rate of oxygen uptake, both initial and final, was higher in its absence. With pyruvate as substrate, both the initial stimulation of the uptake of oxygen and the reduction of the coenzymes were markedly decreased by omitting magnesium; with succinate, its omission produced only a slight decrease in the extent to which the coenzymes were reduced (compare amounts of reduced nucleotide present at 0 and 15 min. in Tables 5 and 6). Magnesium did not increase the stability of the coenzymes during incubation.

Amounts of pyridine nucleotides found in isolated mitochondria

After the investigations described above, all isolations of mitochondria were carried out in 0·25 M-sucrose plus 25 mM-nicotinamide, and all the suspensions were prepared in Chappell–Perry medium plus nicotinamide (25 mM), hereafter called 'saline medium', by the procedure described in the Methods section. The results of analyses of eight different mitochondrial suspensions prepared by this procedure are given in Table 7. The data refer to the original level of the coenzymes in mitochondria isolated and maintained at 0°, and are expressed on the basis of both dry weight and protein content. There was more diphosphopyridine nucleotide than triphosphopyridine nucleotide, and more variability in the amounts of the latter. The levels of DPNH and TPN^+ were least variable. The values for the ratio DPNH/DPN^+ ranged from 0·4 to 1·1 (mean 0·62) and for TPNH/TPN^+ from 1·8 to 7·5 (mean 4·12). These values were all obtained from preparations used subsequently for the metabolic experiments described in this paper (contrast those used by Birt & Bartley, 1960*a*) and include particles derived from two fractions which appear in the 'mitochondrial' pellet after centrifuging in a sucrose–nicotinamide medium. The upper layer, lighter in colour and less firmly packed, resembled the 'fluffy layer' usually obtained in sucrose media but was more copious than in preparations made in the sucrose medium alone. In the routine isolation of mitochondria much, but not all, of the 'fluffy layer' was discarded. In one experiment a rigorous separation of the two fractions ('fluffy layer' and 'mitochondria') was carried out and the coenzyme levels were determined. The 'fluffy layer' contained less total pyridine nucleotide/mg. dry wt. but the proportions of the individual coenzymes were the same as in the mitochondrial fraction. The percentage protein of the two fractions was identical (66 % of the dry matter).

Table 7. *Amounts of pyridine nucleotides in isolated rat-liver mitochondria*

Values are given as (i) μm-moles/mg. dry wt. and (ii) μm-moles/mg. of protein. The first series are the means of eight determinations ± S.E.M. The mitochondrial suspensions analysed contained 20–30 mg. of dry matter/ml.; the percentage of protein in the dry matter was 66 %. In this and subsequent Tables, DPN represents $DPN^+ + DPNH$ and TPN represents $TPN^+ + TPNH$. See also protocol to Table 1.

	(i)	(ii)
DPN^+	1·25 ± 0·03	1·9
DPNH	0·78 ± 0·01	1·2
DPN	2·03 ± 0·06	3·1
TPN^+	0·33 ± 0·01	0·5
TPNH	1·36 ± 0·09	2·1
TPN	1·69 ± 0·11	2·6
Total	3·72 ± 0·24	5·7
$\frac{DPNH}{DPN^+}$	0·62	—
$\frac{TPNH}{TPN^+}$	4·12	—
$\frac{PNH}{PN^+}$	1·35	—

Leakage of pyridine nucleotides from mitochondria suspended in 'saline medium'

The leakage of pyridine nucleotides from the mitochondria in a number of different incubated suspensions is shown in Table 8. In all but one experiment (Expt. 6) most of the coenzyme was retained by the mitochondria (or the particles of 'fluffy layer', Expt. 5) during periods of up to 1 hr. The released nucleotides were nearly all in the oxidized form, and were predominantly diphosphopyridine nucleotides. The exceptional experiment, an incubation for 1 hr. at 30° (Expt. 6), resulted in

Table 8. *Leakage of pyridine nucleotides from mitochondria in 'saline medium' without substrate*

For details of the measurement of the distribution of pyridine nucleotides between mitochondria and the suspending medium see text. Temperature of incubation in Expts. 1–5 was 25° and in Expt. 6 30°. See also protocol to Table 1.

Expt. no.	Time (min.)	PNH/PN+ of whole suspension	PN (μm-moles/ml. of suspension) 'In' mitochondria PN+	PNH	PN	In supernatant fluid PN+	PNH	PN	Percentage in supernatant fluid
1	15	1·7	32	60	92	3·2	0	3·2	3
	45	0·4	47	19	66	5·6	0·5	6·1	8
2	15	1·7	32	59	91	3·2	0	3·2	3
	45	0·4	47	19	66	5·6	0·4	6·0	8
3	50	0·46	28	21	49	4·7	0	4·7	9
4	45	0·46	35	29	64	9·9	0	9·9	13
5	15	—	—	—	—	4·6	0	4·6	—
5*	15	1·7	14	24	38	2·4	0	2·4	6
6	0	1·9	25	53	78	3	0	3	4
	60	0	2	0	2	47	0	47	96

* 'Fluffy layer.'

Table 9. *Leakage of pyridine nucleotides from mitochondria in 'saline medium' without substrate*

Temperature of incubation, 25°; for details of the measurement of the distribution of pyridine nucleotides between mitochondria and the suspending medium, see text. Uptake of O_2 was measured as μl. of O_2/mg. dry wt./hr. See also protocol to Table 1.

Time (min.)	PNH/PN+	PN (μm-moles/ml. of suspension) 'In' mitochondria PN+	PNH	PN	In supernatant fluid PN+	PNH	PN	Percentage in supernatant fluid	Uptake of O_2
0	2·8	24	69	93	0·8	0	0·8	1	0
7	1·5	24	51	75	11	0	11	13	4·5
18	1·7	16	52	68	17	4	21	24	3·8
31	1·0	16	37	53	25	3	28	35	2·5
61	0·15	0·2	3	3·2	53	5	58	95	2·0

Table 10. *Influence of substrates and inhibitors on leakage of pyridine nucleotide from mitochondria*

Temperature of incubations, 25°; concn. of pyruvate and succinate, 10 mM; amytal, 1 mM; 2:4-dinitrophenol, 0·1 mM. The mitochondrial suspensions used in each experiment were derived from the same homogenate. See also protocol to Table 1.

Expt. no.	Substance added	PN in supernatant fluid after 1 hr. (μm-moles/ml.) PN+	PNH	PN	Percentage of PN in supernatant fluid
1	Nil*	34	0	34	59
	Nil†	21	2	23	40
2	Nil	3·4	0·4	3·8	9
	Pyruvate	2·5	0·4	2·9	6
	Succinate	1·4	0·5	1·9	4
2‡	Nil	2·4	0	2·4	6
	Pyruvate	3·0	0	3·0	7
	Succinate	2·1	0	2·1	5
3	Nil	9·9	0	9·9	13
	Succinate	2·7	1·8	4·5	6
4	Nil	4·7	0	4·7	9
	Amytal	6·2	0	6·2	13
	2:4-Dinitrophenol	2·4	0	2·4	5

* Mitochondria prepared and incubated in 'saline medium'; final value for the ratio PNH/PN+ of whole suspension, 0·07.

† Mitochondria prepared and incubated in medium without EDTA as in Table 4; final value for the ratio PNH/PN+ of whole suspension, 0·46.

‡ 'Fluffy layer'.

the almost complete release of the coenzymes from the mitochondria, and, at the same time, a very low final amount (below the limit of detection) of reduced nucleotide in the whole suspension. Infrequently, a similar leakage of pyridine nucleotides was obtained after incubation at 25°, and again the final ratio of reduced coenzyme to oxidized coenzyme in the whole suspension was very low. In the experiment of this kind described in Table 9, 35 % of the pyridine nucleotide was lost from the mitochondria in the first 30 min., while the ratio of reduced coenzyme to oxidized coenzyme in the whole suspension fell to one-third of its original value; in the last half-hour, a further 60 % of the coenzyme leaked out, and the ratio decreased to one-eighteenth of its original value. The respiration rate declined steadily during the entire incubation. It is noteworthy that small amounts of reduced pyridine nucleotide were detected in the incubating medium (compare Birt & Bartley, 1960*a*).

These experiments suggested a relationship between the maintenance of reduced nucleotide in the mitochondria and the rate of leakage. Further investigation (Table 10) showed that this relation was not a simple one, for the addition of a substrate which increased the amount of reduced coenzyme (e.g. pyruvate or succinate, Table 10) and of 2:4-dinitrophenol, which decreased the amount to the limits of detection, both apparently diminished the rate of leakage. EDTA, in Expt. 1 (Table 10) had decreased the ability of the mitochondria to maintain the level of reduced coenzymes throughout an incubation of 1 hr. without added substrate (compare Table 4), whereas amytal had at first increased then decreased this level; but both accelerated the release of coenzymes. Direct comparison of the leakage from mitochondria incubated alone and with 2:4-dinitrophenol or amytal showed that the increased rate of loss due to amytal occurred during the period of enhanced oxidation of the reduced nucleotide (the second 30 min. in Table 11); the slight decrease in the amount of coenzyme lost by leakage with 2:4-dinitrophenol also occurred in the second 30 min. of the incubation.

Reactivity of pyridine nucleotides in the supernatant fluid

Nucleotides leaking from mitochondria into the supernatant fluid can act as hydrogen acceptors when substrate is added. In the experiment described in Table 12, two samples of the same mitochondrial suspension were shaken without substrate for 28 min., one at 0° and the other at 25°, and the distribution of the pyridine nucleotides between supernatant fluid and the mitochondria was determined for each sample. Pyruvate plus malate or β-hydroxybutyrate were added to

Table 11. *Influence of 2:4-dinitrophenol and amytal on the leakage of pyridine nucleotides from mitochondria*

All values for pyridine nucleotide levels are given as μm-moles/ml. Temperature of incubation, 25°. See also protocol to Table 1.

Time (min.)		No addition				2:4-Dinitrophenol (0·1 mM)				Amytal (mM)			
		PN^+	PNH	PN	PNH PN^+	PN^+	PNH	PN	PNH PN^+	PN^+	PNH	PN	PNH PN^+
30	Whole suspension	43	18	61	0·42	48	0·5	48·5	0·01	34	28	62	0·82
	Supernatant fluid	3·8	0	3·8	—	4·0	0	4·0	—	5·2	0	5·2	—
	Mitochondria	39	18	57	0·46	44	0·5	44·5	0·01	29	28	57	0·97
	Percentage in supernatant fluid	9	0	6	—	8	0	8	—	15	0	8	—
60	Whole suspension	35	20	55	0·57	44	1	45	0·02	49	0·5	49·5	0·01
	Supernatant fluid	5·3	0	5·3	—	4·9	0	4·9	—	11	0	11	—
	Mitochondria	30	20	50	0·67	39	1	40	0·03	38	0·5	38·5	0·01
	Percentage in supernatant fluid	15	0	10	—	11	0	11	—	22	0	22	—

Table 12. *Reactivity of pyridine nucleotides released from mitochondria*

All values for pyridine nucleotide levels are given as μm-moles/ml. Concn. of β-hydroxybutyrate, pyruvate and malate, 10 mM. See also protocol to Table 1.

Addition	Temp. of pre-incubation or incubation	Time (min.)	Whole suspension					Supernatant fluid					Mitochondria				
			PN+	PNH	PN	PNH/PN+	Loss (%)	PN+	PNH	PN	PNH/PN+	Loss (%)	PN+	PNH	PN	PNH/PN+	Loss (%)
Nil	0°	0	20·5	48	68·5	2·3	—	—	—	—	—	—	20·5	48	68·5	—	—
Nil	0	28	24	46	70	1·9	0	10·4	0	10·4	0	—	13·6	46	59·6	3·4	—
Nil	25	28	30	26·5	56·5	0·9	18	10·1	0·9	11·0	0·09	—	19·9	25·6	45·5	1·3	24
β-Hydroxybutyrate	25 (from 0°)	43	24	40·5	64·5	1·7	6	9·4	0·9	10·3	0·10	—	14·6	39·6	54·2	2·7	9
Pyruvate plus malate	25 (from 0°)	43	17	45	62	2·7	9	3·7	6·9	10·6	1·9	—	13·3	38·1	51·4	2·9	14
β-Hydroxybutyrate	25 (from 25°)	43	22	25	47	1·1	31	9·5	1·3	10·8	0·14	—	12·5	23·7	36·2	1·9	39
Pyruvate plus malate	25 (from 25°)	43	18	42	60	2·3	12	6·5	4·3	10·8	0·66	—	11·5	37·7	49·2	3·3	17

portions of each of the pre-incubated suspensions, the mixtures were incubated for 15 min. at 25° and the distribution of the nucleotides between supernatant fluid and mitochondria was again determined. The addition of pyruvate plus malate resulted in a hydrogenation of the coenzymes in the supernatant fluid, more especially when the pre-incubation had been at 0°. The amounts of pyridine nucleotide released into the supernatant fluid during pre-incubation for 28 min. without substrate were the same at both 0° and 25°; there were no great changes in these amounts during the 15 min. of incubation with added substrate, though the levels of total nucleotide in the whole suspensions declined by up to 13%.

It is apparent that ratios of reduced to oxidized mitochondrial nucleotide assessed by analysis of whole suspensions of mitochondria will be in error because of leakage from the particles during incubation. Usually, the ratios measured in this way will be lower than the true values, because the leakage of oxidized coenzyme exceeds that of reduced coenzyme. This can be seen by comparing ratios of reduced to oxidized nucleotide for 'Whole suspension' and 'Mitochondria' in Tables 11 and 12.

Pyridine nucleotides of mitochondria isolated from rat brain

The applicability of the methods for extraction and estimation of pyridine nucleotides, and the amounts and susceptibility to experimental control of the oxidized and reduced coenzymes of the mitochondria of rat-brain tissue, have been examined briefly. The total amount of pyridine nucleotide found in isolated brain mitochondria was considerably lower than that found in liver mitochondria (compare Tables 7 and 13): there was approximately one-third of the amount of diphosphopyridine nucleotide and one-fiftieth of the amount of triphosphopyridine nucleotide. The initial proportion of reduced coenzymes was extremely low, both DPNH and TPNH occurring at the limit of detection in a suspension containing 20 mg. dry wt. of mitochondria/ml.

During the incubation of brain mitochondria in 'saline medium' alone there was little change in the proportion of reduced coenzyme, but considerable loss (30%) of the total pyridine nucleotide occurred. The incubation of brain mitochondria for 15 min. with succinate, glutamate, pyruvate or α-oxoglutarate demonstrated marked differences in the ability of these compounds to affect the ratio of reduced to oxidized coenzymes (Table 14). The level of reduced coenzymes was greatly increased by succinate and glutamate, but was not altered by either pyruvate or α-oxoglutarate.

Table 13. *Levels of endogenous pyridine nucleotides in isolated brain mitochondria*

All values for pyridine nucleotide levels are the means of the analyses of three preparations of mitochondria; they are given as μm-moles/mg. dry wt.; the suspensions of mitochondria contained 20–30 mg. of dry matter/ml. See also protocol to Table 1.

DPN^+	DPNH	DPN	TPN^+	TPNH	TPN	$\frac{PNH}{PN^+}$
0·62	0·05	0·67	0·01	0·02	0·03	0·11

Table 14. *Effect of added substrates on the pyridine nucleotides of brain mitochondria*

All values for pyridine nucleotide levels are given as μm-moles/mg. dry wt. Temperature of incubation, 25°; concn. of pyruvate, glutamate, α-oxoglutarate and succinate, 10 mM. See also protocol to Table 1.

Substrate added	Time (min.)	PN^+	PNH	PN	$\frac{PNH}{PN^+}$
Nil	0	0·65	0·11	0·76	0·17
Nil	15	0·44	0·09	0·53	0·20
Pyruvate	15	0·45	0·08	0·53	0·18
Glutamate	15	0·42	0·22	0·64	0·52
α-Oxoglutarate	15	0·54	0·10	0·64	0·19
Succinate	15	0·29	0·27	0·56	0·93

Table 15. *Comparison of the pyridine nucleotide content of mitochondria prepared for sampling by two different methods*

Values under (i) are taken from Birt & Bartley (1960*a*) and those under (ii) are from this paper (Table 7). Values for DPN ($DPN^+ + DPNH$) and TPN ($TPN^+ + TPNH$) are given as μm-moles/mg. of protein.

	(i)	(ii)
DPN	3·7	3·1
TPN	3·6	2·6
$\frac{DPNH}{DPN^+}$	0·22	0·62
$\frac{TPNH}{TPN^+}$	0·86	4·12

DISCUSSION

Two series of values obtained in investigations described in this and a preceding paper (Birt & Bartley, 1960*a*) for the amounts of pyridine nucleotides in isolated mitochondria are given in Table 15. All the suspensions used contained nicotinamide, which decreases the losses of coenzyme occurring during the isolation even at 0°. For the first series, mitochondrial preparations containing relatively large amounts of 'fluffy layer' were analysed; the sampling of these suspensions involved a short time (2 min.) at a temperature of about 10° (when the substrate was added and mixed) and was therefore somewhat slower than in the experiments from which the second series was derived. Hence the lower total amount of coenzyme/mg. dry wt. in the former series. In both series the variability in the level of triphosphopyridine nucleotide was greater that than of the diphosphopyridine nucleotide perhaps because of the greater permeability of the mitochondrial membrane to the former (Birt & Bartley, 1960*a*).

The two series also showed markedly different proportions of oxidized and reduced coenzyme; the mean values of the ratios $DPNH/DPN^+$ and $TPNH/TPN^+$ were 0·62 and 4·12 (series 1) and 0·22 and 0·86 (series 2) respectively. The values shown in Table 15 were obtained (i) by analyses of resuspended mitochondrial pellets and (ii) by analyses of the original mitochondrial suspensions. The ratio $TPNH/TPN^+$ was much greater than the ratio $DPNH/DPN^+$ in both these analyses, but the constituents of the media used for isolation and suspension may influence the relative proportions of oxidized and reduced coenzymes greatly. For example, both nicotinamide and EDTA (in the sucrose or 'saline' media) decreased the ratio of reduced to oxidized pyridine nucleotide. It has been shown previously that this ratio can be altered also by varying the method of preparation of the particles (see Birt & Bartley, 1960*a*). Thus the precise significance of the proportions of reduced coenzyme in freshly prepared mitochondria cannot be established without consideration of the preparative procedure used.

Stability of pyridine nucleotides during incubation

Although the pyridine nucleotides of mitochondria are more stable than those of whole-tissue preparations (compare Bassham *et al.* 1959) in the great majority of experiments there was some loss of coenzyme during both the isolation and incubation for 1 hr. at 25°. In the early stages of the isolation, before the washing of the particles was completed, the rate of loss was rapid (compare values for 'absolute loss', Table 1) and it is likely that much of the destruction was due to the

extremely active nucleotide-splitting enzymes of contaminants of the mitochondrial fraction, especially the microsomes; nicotinamide, which inhibits DPN^+ nucleosidase, an enzyme of relatively low activity in the mitochondria themselves (Jacobson & Kaplan, 1957*a*), was most effective at this time. Mitochondria can lose pyridine nucleotides to the suspending medium, even at 0° (see Table 12), and a vigorous destruction of the coenzymes in the supernatant fluid may result in continuing leakage and therefore in an appreciably lower total level of coenzyme in the original whole suspension. It is possible that during the subsequent manipulations the mitochondrial nucleotide-splitting enzymes are themselves responsible for much of the loss that occurs, because (i) the rate of destruction at 25° was relatively low, and was less readily influenced by nicotinamide, (ii) in the washed suspensions in 'saline' little of the pyridine nucleotide was initially extramitochondrial, though there was always some leakage, and (iii) even when there was appreciable destruction in the whole suspension, the level in the supernatant fluid remained approximately constant (see Tables 11 and 12). The supernatant fluid may, however, retain some capacity for destroying coenzymes. Added pyridine nucleotides disappear at a low rate (Birt & Bartley, 1960*a*) and when the leakage of coenzymes from the mitochondria was very pronounced the rate of destruction was sometimes increased (Table 9, compare the first 31 and the last 30 min.).

The extent to which added substrates protected the coenzymes was variable. In nine out of 15 experiments there was a decrease in the rate of destruction after the addition of substrates with DPN-dependent dehydrogenases, though a similar effect was seen in only three out of eight experiments with succinate, and not at all in the one experiment with choline. The protective action may be due to (i) decreased leakage of coenzymes, (ii) an increase in the level of reduced coenzymes, which are relatively more stable (the explanation given for a similar effect of substrate in minces and homogenates), or (iii) a more rapid turnover of the coenzymes during the accelerated operation of the electron-transport chain itself. Although the first factor may be of some importance it does not appear to be a sufficient explanation, because amytal can increase the leakage without increasing the destruction of coenzymes, and succinate consistently decreased the leakage but not the destruction. Moreover 2:4-dinitrophenol, apparently without effect on leakage, increased the rate of destruction, though whether the loss occurred before or after the release of coenzyme from the mitochondria was not determined. The second and third factors, however, both appeared to influence the stability of the coenzymes. Succinate, which was capable of reducing a greater proportion of the pyridine nucleotides than any other substrate tested, was less effective in inhibiting the destruction of the coenzymes than was pyruvate (the protective action was shown in six out of 12 and 10 out of 12 experiments respectively). On the other hand, losses produced by 2:4-dinitrophenol (see Birt & Bartley, 1960*b*) were seen more commonly with added pyruvate as substrate (in 10 out of 12 experiments) than with succinate (in 0 out of 12 experiments); 2:4-dinitrophenol stimulated the respiration with pyruvate less than with succinate, but lowered the level of reduced pyridine nucleotide more rapidly with the former. Thus it seems that the rapid passage of electrons along the complete respiratory chain from DPNH to oxygen together with a maintained high level of reduced pyridine nucleotide is necessary for the maximum preservation of the coenzymes. These conditions were fulfilled when there was an excess of substrate oxidized by pyridine nucleotide-dependent dehydrogenases. The coenzymes of the mitochondria would then be fully utilized in binding the substrates and accepting hydrogen, and especially in the presence of nicotinamide, the ability of the splitting enzymes to compete successfully for the nucleotides would be greatly impaired.

Effect of constituents of the isolation and incubation media on pyridine nucleotides

Nicotinamide was always added to the media used for isolation and incubation. The following effects on the mitochondria were observed: (i) The sedimentation characteristics of the particles were altered, and the proportion of 'fluffy layer' increased; when the mitochondria were exposed to nicotinamide in the sucrose only, they acquired and retained sufficient of it to influence their subsequent reactions, even when the 'saline medium' had no nicotinamide. (ii) The destruction of the coenzymes, particularly the oxidized forms, was decreased (compare Bassham *et al.* 1959). (iii) The reduction of pyridine nucleotides by endogenous but not added substrates (at 10 mM concentration) was prevented. (iv) The rate of endogenous respiration was lowered by 30–50 % but that with added substrate was decreased very little (compare Bassham *et al.* 1959).

The mechanism of the action of nicotinamide (and other compounds) as inhibitors of nucleosidases has been studied by Kaplan *et al.* (Zatman, Kaplan & Colowick, 1953; Zatman, Kaplan, Colowick & Ciotti, 1954), who have shown that it replaces the OH^- ion of water as an acceptor for the adenine–ribose–phosphate–phosphate–ribose group, i.e. it can be bound on an enzyme surface at a site normally occupied by the nicotinamide

residue of the oxidized coenzyme. It seems likely that all the observations listed above can be explained by assuming that nicotinamide is bound in this way at the reactive sites of other enzymes. Thus it may be retained by washed mitochondria, attached to and influencing the action of the enzymes concerned with the oxidation and reduction of pyridine nucleotides. Normally, with endogenous substrate, the dehydrogenase systems are not saturated, for both the level of reduced coenzyme and the oxygen consumption increase greatly when substrates are added. The coenzymes, as their turnover in the cyclic oxidation–reduction processes is not proceeding at its maximum rate, can be replaced at the binding sites by nicotinamide, so that the proportion of reduced coenzymes and the endogenous respiration are both diminished. When substrate is in excess, the opportunities for nicotinamide to replace the bound coenzyme will be greatly decreased, and the inhibitor has no significant influence on either the reduced coenzyme or the respiratory rate. Jacobson & Kaplan (1957*a*) have shown that nicotinamide depresses the rate of oxidation of extramitochondrial DPNH, and this, too, is probably due to the binding of the inhibitor at sites normally reacting with pyridine nucleotide.

Adenosine triphosphate was almost without effect on the pyridine nucleotides, but apparently preserved the mitochondria from changes regarded as degenerative (see Birt & Bartley, 1960*b*).

Magnesium added to mitochondrial suspensions facilitates the coupling of oxidation and phosphorylation (Chance & Williams, 1956) and its omission may result in a rapid increase in the rate of oxygen uptake after incubation for a few minutes (Baltscheffsky, 1957). Two separate effects of magnesium on mitochondria in the 'saline medium' were observed. One, evident when no substrate was added, was to promote the oxidation of the reduced pyridine nucleotides; in its absence, the increased oxygen consumption (cf. Baltscheffsky, 1957) did not result in the usual progressive oxidation of the coenzymes (Tables 5 and 6). The second effect of magnesium was to facilitate the reduction of pyridine nucleotides and to permit the stimulation of oxygen uptake by added pyruvate (Table 5), probably because pyruvic decarboxylase requires magnesium for its maximum activity (Green, 1952); on the other hand, magnesium had little effect on the behaviour of mitochondria incubated with succinate, though neither the increase nor the decline in the ratio of reduced to oxidized pyridine nucleotide was as pronounced when it was omitted (Table 6). From all these data, it appears that magnesium is necessary for the oxidation of DPNH by the respiratory chain, perhaps being required for the binding of some mitochondrial component essential for oxidative phosphorylation (see Kielley & Bronk, 1957; Linnane & Ziegler, 1958). The most striking feature of the experiments without added magnesium was the length of time during which the reduced coenzymes were retained. This effect was not observed in any other experiments at 25° and suggests that the linkage between DPNH (and therefore TPNH; see Birt & Bartley, 1960*b*) and all the oxidative pathways has been broken; Green (1959) has reported that magnesium is required for the binding of DPN^+ by mitochondria, and the EDTA of the suspending medium may remove sufficient of the endogenous magnesium to destroy the binding sites involved. There are a number of conflicting reports about the influence of added EDTA on suspensions of mitochondria (for summary, see Schneider, 1959). There was no evidence for a degeneration of the particles incubated in the media without EDTA. Its addition will lower slightly the concentration of free magnesium in the 'saline medium'; hence, as pyruvate oxidation requires magnesium, the ability of pyruvate to reduce the coenzymes may be decreased and the oxygen uptake with this substrate diminished. However, as it did not prevent the oxidation of reduced nucleotides when no substrate was added (though it retarded it appreciably) there was still sufficient free magnesium in the medium for the operation of the electron-transport chain, which also requires magnesium. Thus the concentration of magnesium needed to activate this chain may be lower than that required for maximum activity of pyruvic decarboxylase.

The results obtained in this investigation of the influence of various constituents of the suspending medium emphasize the dependence of the proportions of oxidized and reduced coenzyme in isolated mitochondria on the method of preparation. Thus two of the components of the 'saline medium', nicotinamide and EDTA, lowered the original level of reduced coenzymes and decreased the extent to which reduction occurred during incubation. The addition of nicotinamide, especially, may account for much of the discrepancy between the ratios of reduced to oxidized coenzyme measured by Chance & Williams (1955) and those reported here. Differences in the time elapsing before measurements are made and in the manner of incubating the mitochondria may also be important in this regard.

Leakage of pyridine nucleotides from mitochondria during incubation

The leakage of pyridine nucleotides from mitochondria incubated under various conditions has been described by several workers and investigated by direct measurement of the coenzymes by Kaufman & Kaplan (1959). They report an almost

complete loss of nucleotide during the incubation of mitochondria at 30° in 0·25M-sucrose and 0·02M-phosphate. Nicotinamide did not affect the leakage, but substrates, especially succinate, decreased it. The leakage of coenzymes from mitochondria incubated in the 'saline medium' at 25° without added substrate was much less complete. This was probably due to several factors, namely, the difference in temperature (in the one experiment at 30° in 'saline medium' leakage was extremely rapid, though increasing the temperature from 0° to 25° had little effect on the loss), the low concentration of inorganic phosphate and the presence of ATP and magnesium. EDTA, which assists in the retention of coenzymes by mitochondria incubated at 30° in 0·25M-sucrose, even after almost complete oxidation of the nucleotides (Lester & Hatefi, 1958), had the reverse effect as a component of the 'saline medium'. It is possible that EDTA, by efficiently binding calcium, which is present in isolated mitochondria at the same concentration as magnesium (Bartley & Amoore, 1958), alters the mitochondrial structure sufficiently to permit a redistribution of the pyridine nucleotides between the mitochondria and the suspending medium. Occasionally, substantial leakage occurred in the 'saline medium', but only in combination with other changes interpreted as indicating the breakdown of the mitochondria. The ability of added substrates to diminish the leakage was also observed in this medium, but an apparently similar effect of 2:4-dinitrophenol may be due to an accelerated destruction of the coenzymes in the supernatant fluid, especially during the early stages of the incubation (see Table 11). In this experiment (Table 11), if the difference between the amounts of total pyridine nucleotide in the whole suspensions with and without 2:4-dinitrophenol was due to destruction of released coenzymes, the leakage in 2:4-dinitrophenol during 30 min. was about 28% of the total rather than 8%. Amytal always increased the leakage after 1 hr.

These results are in agreement with the conclusions of Lester & Hatefi (1958) and Corwin & Lipsett (1959), that the oxidation of the pyridine nucleotides is not the sole factor controlling leakage, though the nearer to completion this oxidation becomes (i.e. when the ratio of reduced to oxidized coenzyme falls below 0·1) the more rapid is the loss. The degenerative changes occurring in mitochondria with low levels of reduced nucleotide (see Birt & Bartley, 1960*b*) may include the oxidation of some mitochondrial component necessary for the binding of the coenzyme, as supposed by Lester & Hatefi (1958); also the breakdown of mitochondrial 'compartmentation' may increase the accessibility of the coenzyme binding-sites to known competitors for attachment, e.g., ATP, calcium and phosphate.

The difference in the ability of β-hydroxybutyrate and pyruvate plus malate to reduce the pyridine nucleotides liberated into the suspending medium may be due to the difference in the firmness of the binding of the dehydrogenases to the mitochondrial structure. Unlike β-hydroxybutyric dehydrogenase, the enzymes responsible for dehydrogenating pyruvate plus malate were less active if the pre-incubation was at 25° rather than at 0°. It is likely that the reduction of the coenzymes in the supernatant fluid was due mainly to liberated malic dehydrogenase, which may undergo partial denaturation during the shaking for 28 min. at 25° without substrate.

Pyridine nucleotides of mitochondria of other tissues

A short study of the coenzymes in the mitochondria of other tissues gave an indication of the general applicability of the method of analysis to the study of mitochondrial nucleotides. No difficulties were found with the two preparations analysed. Mitochondria from the thoracic muscle of the fly *Phormia terraenovae* contained no detectable pyridine nucleotide [for other estimates of the coenzyme content of insect-muscle mitochondria see Chance & Sacktor (1958); Price & Lewis (1959)]. Rat-brain mitochondria, however, contained DPN^+ and small amounts of DPNH, TPN^+ and TPNH. The values obtained were in fair agreement with those of Jacobson & Kaplan (1957*b*) and showed that the ratio of reduced to oxidized coenzymes in these mitochondria is extremely low, though it could be increased by incubation with a number of substrates, especially succinate. In view of the proposed role for triphosphopyridine nucleotide in rat-liver mitochondria (Birt & Bartley, 1960*b*) it is of interest that the brain particles, which have a low transhydrogenase activity (Vignais & Vignais, 1957; Humphrey, 1957), have a very low concentration of triphosphopyridine nucleotide also.

SUMMARY

1. The amounts and behaviour of the pyridine nucleotides of rat-liver mitochondria are shown to depend on the method of preparation and the constituents of the suspending medium. In the present work, the tissue was fractionated in a sucrose–nicotinamide medium and the mitochondria were incubated in a 'saline medium'.

2. The rate of loss of the mitochondrial pyridine nucleotide was about one-tenth of that of the coenzymes of the mince from which the particles were isolated.

3. Nicotinamide increased the initial amounts and stabilized the pyridine nucleotides of the

isolated mitochondria during incubation at 25°. The endogenous respiration and the capacity of the particles to maintain reduced coenzyme were both decreased. With nicotinamide the proportions of reduced coenzyme produced by succinate and pyruvate were lowered, but the respiration rates were unaltered.

4. Ethylenediaminetetra-acetic acid decreased by about 7% the initial proportions of reduced coenzyme and diminished the reduction of the nucleotides by both endogenous substrate and added pyruvate. The rate of destruction was increased about fourfold by ethylenediaminetetra-acetate, and the respiration rate, though initially lower, was also more stable.

5. Magnesium was required for the oxidation of reduced pyridine nucleotide during incubation without added substrate and for the reduction of the coenzymes by added pyruvate. The initial rate of respiration without added substrate and with pyruvate were lower by about 25% when magnesium was present.

6. Analyses of freshly isolated mitochondria at 0° showed that the ratio of reduced diphosphopyridine nucleotide/oxidized diphosphopyridine nucleotide ranged from 0·4 to 1·1 (mean 0·62) and that of reduced triphosphopyridine nucleotide/oxidized triphosphopyridine nucleotide ranged from 1·8 to 7·5 (mean 4·12). The levels of reduced diphosphopyridine nucleotide and oxidized triphosphopyridine nucleotide were least variable. The 'fluffy layer' of these preparations contained less total pyridine nucleotide/mg. dry wt., but the same proportion of each individual nucleotide as the rest of the particles.

7. The rate of leakage of coenzymes from the mitochondria at 25° was usually low, about 10% in 1 hr.; it was increased (up to ninefold) when there was considerable oxidation of nucleotides. Added pyruvate and succinate decreased the leakage occurring in 1 hr. but amytal and ethylenediaminetetra-acetic acid increased it. The released pyridine nucleotide was reduced by pyruvate plus malate and β-hydroxybutyrate.

8. Rat-brain mitochondria had lower levels of all the coenzymes, particularly of the triphosphopyridine nucleotides. The initial ratio of reduced coenzyme to oxidized coenzyme was relatively low (0·11–0·17) and was increased by incubation with succinate and glutamate (up to sixfold), but not with pyruvate or α-oxoglutarate.

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Some Experiments on Intrinsic Factor

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Latner, Merrills & Raine (1956) reported that an apparently homogeneous preparation of intrinsic factor from dried pig-stomach powder could be resolved into an active and an inactive fraction by chromatography on the cationic-resin Amberlite IRC-50. The inactive ('peak 1') material, moreover, inhibited absorption of cyanocobalamin in the normal rat.

Pyloric secretion, from a blind pyloric pouch in the pig, freed from muco-substance by filtration had been shown (Heatley *et al.* 1954) to possess intrinsic factor activity in the patient with pernicious anaemia in remission, and since a supply of this material in the fresh state was available it was decided to see whether a concentrate from it could be fractionated by the chromatographic process of Latner *et al.* (1956); it was hoped that a purer final product might be obtained from the secretion than from the mucosal starting materials generally used. It was also hoped to examine the part played by the inactive 'peak 1' of pig and human material in the lack of response when crude preparations from these sources are tested in gastrectomized rats. Results of some interest along these and other lines have been obtained; though incomplete they are presented now since dispersal of the authors precludes further work at the present time.

MATERIALS AND METHODS

Measurement of intrinsic-factor activity. The earlier tests were done on patients with pernicious anaemia in remission, by using radioactively labelled vitamin B_{12}, and collecting faeces in cardboard cartons. The radioactivity was measured by placing the unopened cartons in turn within a ring of Geiger counters and making suitable geometrical corrections (Bradley, 1957); the unpleasant homogenizing and sampling of the faeces was thus avoided. Towards the end of the work tests were made as well on gastrectomized rats by the method of Taylor, Mallett, Witts & Taylor (1958), activity in 8-day collections of faeces being measured in a scintillation counter.

For the human experiments [^{60}Co]vitamin B_{12} of specific activity about 0·3 μc/μg. was used, and, for the rat tests, vitamin labelled with ^{58}Co having a specific activity of 1·0–3·2 μc/μg. Dilutions of these materials were stored at 4° and fresh batches of the ^{58}Co labelled material were obtained from The Radiochemical Centre, Amersham, Bucks., each month to reduce the risk of deterioration.

Human gastric juice. This was obtained partly from the authors and partly from patients undergoing investigation of gastric function. All subjects were fasting; juice from some was collected after stimulation by a maximal dose of histamine (Kay, 1953). Only juice containing free hydrochloric acid was used. The different products were not prepared separately.

Pig-mucosal-powder concentrate. This material had been kindly supplied by Dr L. Ellenbogen of Lederle Laboratories and was a concentrate (45774–133) prepared by ammonium sulphate precipitation of an aqueous extract from desiccated and defatted hog pylorus.

Pig pyloric secretion. The pig was one of a number prepared with gastric or duodenal fistulae by Professor Florey and Dr M. A. Jennings. It had a blind pyloric pouch, opening to the surface, which had been formed when it was a few weeks old (Jennings, Florey, Stehbens & French, 1960). Even shortly before the pig's death, 7 years and 2 months later, it was producing juice of the usual appearance and consistency of pyloric-fistula juice, and as the pouch had grown with the pig the rate of secretion was over 250 ml. in 12 hr. Pouches of this kind rarely remain usable, for one reason or another, for more than 6 months. Pyloric secretion was collected in a special Perspex container (devised by Mr J. Kent) which was applied in a broad bandage while the pig was feeding and was left in position for several hours. To reduce bacterial action, 20 mg. each of penicillin and streptomycin sulphate were put in the container before it was fixed to the pig. The collecting pot was not kept on continuously, and since the pig was so large (its weight some months before death was estimated at 200 kg.) the pot could be put in position only when the pig would co-operate, and only a part of the total juice secreted was actually collected and used.

Treatment of pyloric secretion. The secretion, faintly alkaline and of the consistency of egg-white, was filtered under suction through a 5-on-3 fritted glass filter (Heatley, 1959), or latterly through an Oxoid membrane laid on such a filter. This had the advantage that, with the help of a strip of cellophan to lift its edge, the membrane could be stripped off and replaced by a fresh membrane, thus avoiding both the need to clean the funnel and the loss of the 5–6 ml. of filtrate left in its pores.

When it had been established that intrinsic-factor activity was adsorbed at pH 4·9 on to Amberlite resin XE-64, whereas pepsinogen passed straight through, the

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routine treatment of the crude secretion was modified as follows: the secretion was brought to about pH 5 by whisking in 1·1 ml. of N-acetic acid/100 ml. before placing on the filter. Attached to the stem of the latter, as shown in Fig. 1, was a small column of resin XE-64 previously equilibrated with the 'buffer A' of Latner *et al.* (1956) and rinsed just before use with buffer A from which gas had been removed. The polythene tube allowed the air pressure over the column to equalize with that in the outer flask when this was evacuated or filled with air at the completion of filtration. The rate of flow through the columns varied, but if it was less than that of filtration (as happened with most columns initially) the level of fluid in the stem of the funnel would rise and flow rate through the column would be correspondingly increased. When filtration was complete, the small column was detached, well washed with buffer A, then the bound intrinsic factor was eluted by percolation with 0·5 M-phosphate buffer, pH 6·8. Elution was considered to be complete when the pH of the eluent had risen above 6·0.

At this or other stages of purification the active material was concentrated by fully saturating with ammonium sulphate, spinning down the precipitate, usually in a Spinco model L centrifuge, taking it up in the minimum amount of water or 0·01 N-HCl and dialysing exhaustively, first in 0·01 N-HCl and then in water. The granular grey precipitate remaining was removed by centrifuging and the supernatant pooled with other batches and submitted to more detailed fractionation on the same resin.

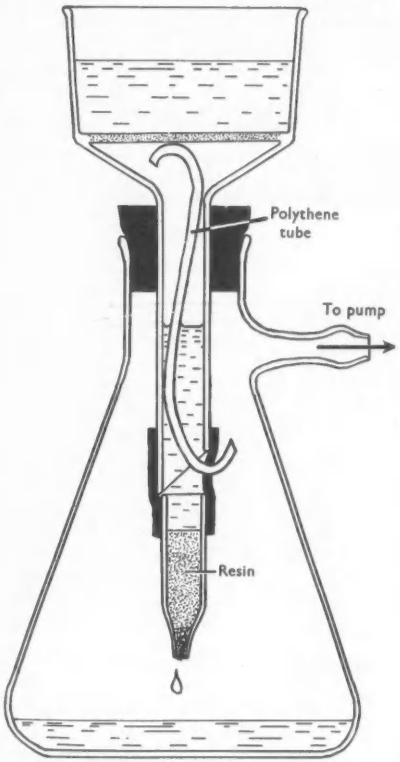

Fig. 1. Arrangement for filtration of pig pyloric secretion and for trapping intrinsic-factor activity in Amberlite resin XE-64. For details see text.

Amberlite resin. Amberlite resin XE-64, a cationic-exchange resin chemically identical with Amberlite IRC-50. Particles passing a 90-mesh sieve were freed from fines and then prepared as described by Latner *et al.* (1956).

Conduct of chromatographic separation. The concentrate, usually well dialysed, was brought to the ionic composition of Latner's buffer A by adding the calculated amounts of sodium phosphate and sodium hydroxide; it was then passed through the column of resin (packed in the usual way) before continuing percolation of the latter with buffer A. The effluent was collected on a fraction cutter and the volume and adsorption at 275 mμ of each fraction or of several, pooled, fractions were measured. Buffer A was replaced by other buffers and further fractions were collected. The whole operation was carried out in a cold room at about 4°.

Electrophoresis. The micro-method of Kohn (1958), with strips of Oxoid membrane filter, was used with dilute nigrosine as stain. The apparatus was copied from a minor modification of Dr R. Consden's apparatus (personal communication). All runs were made at about 4°.

RESULTS

All the tests on pernicious-anaemia patients have been summarized in Table 1 and are individually referred to in the text by the numbers in column 1. Since tests were done singly and on a series of different patients (who could in any case be given no more than six doses of the [^{60}Co]vitamin B_{12}) only a qualitative interpretation of results could be made.

Nearly all the work was done on pig pyloric secretion; that a filtrate of it was still very active was shown by a preliminary experiment (1, Table 1). Fractions of the filtrate precipitated by 25–56 and by 56–100 % saturation with ammonium sulphate were also active (2, 3, Table 1). It was noticed that saturation of the acidified secretion with ammonium sulphate always resulted in a strong odour of caprylic acid.

Precipitation with cetylpyridinium chloride. Small increments of aqueous 1 % cetylpyridinium chloride were added to an exhaustively dialysed ammonium sulphate precipitate of filtrate from pig pyloric secretion (Scott, 1955; Blumberg & Ogston, 1958). The precipitate and supernatant were separately freed from excess of reagent by shaking with chloroform, a process which, applied more vigorously for 30 min. to another sample, did not destroy all activity (4, Table 1). The supernatant remained active (5, Table 1). The precipitate was inactive (6, Table 1) but was so scanty that purification would have been negligible.

Fractionation of pig pyloric secretion on Amberlite resin XE-64. An exhaustively dialysed ammonium sulphate precipitate of filtrate from pig pyloric secretion was passed through a column of resin XE-64 exactly as described by Latner *et al.* (1956). The absorption at 275 mμ of the successive eluate fractions closely resembled that obtained by these authors with a preparation from pig-stomach powder. 'Peak 2' and 'peak 1' were found to have pronounced and slight activity respectively (8, 7, Table 1), though the latter was tested at nearly double the equivalent concentration of the former. Similar 'peak-2' material prepared in the same way on other occasions, after ammonium sulphate precipitation and exhaustive dialysis, was found to give marked activity in the amounts shown in test nos. 11, 12, 14, 17 and 21 of Table 1).

Adsorption of intrinsic-factor activity from crude secretion filtrate. When resin XE-64 had been shown to adsorb activity from concentrates, a test was made to see whether it could do so also from the crude filtrate of pig pyloric secretion. About 90 ml. of filtrate was adjusted to about pH 4·9 with acetic acid before passing it through a small column of the resin. After brief washing of the column with buffer A, the eluate with buffer B was tested and found to be highly active (9, Table 1). After this, most material was collected in resin at the time of filtration (see Materials and Methods section), the secretion being acidified with 1·1 ml. of N-acetic

Table 1. *Tests of intrinsic-factor activity in pernicious-anaemia patients in remission*

For details of test see text.

Test no.	Patient	Material tested	Wt. tested (mg.)	Equivalent volume of original secretion (ml.)	Percentage of vitamin B_{12} (dose 0·5 μg.) excreted	
					Given alone	Given with test preparation
1	I.G.	Filtrate from pig pyloric secretion dialysed against 0·01 N-HCl and water	—	110	86·7	25·6
2	F.T.	Filtrate from pig pyloric secretion precipitated by 25–56% saturation with ammonium sulphate	11·6	100	85·2	38·8
3	W.J.	As 2, but precipitated by 56–100% saturation	41	250	77·2	22·8
4	L.W.	Redissolved precipitate from ammonium sulphate shaken 30 min. with chloroform	27	100	81·8	52·1
5	L.W.	Well-dialysed concentrate not precipitated by cetylpyridinium chloride	—	100	81·8	66·6
6	L.W.	Precipitate from 5	—	100	81·8	91·6
7	W.R.	'Peak 2' from resin XE-64, from concentrate of pig pyloric secretion	—	109	94·6	49·7
8	W.R.	'Peak 1' from resin XE-64, from concentrate of pig pyloric secretion	—	209	94·6	64·8
9	W.R.	Eluate from resin XE-64, through which crude filtrate from secretion had been passed	—	90	94·6	40·0
10	W.R.	Eluate from charged column with buffer A containing 2 M-NaCl	—	405	94·6	82·1
11	W.R.	'Peak 2' fraction from pig pyloric secretion	—	100	94·6	41·3
12	E.A.	'Peak 2' fraction from pig pyloric secretion	0·49	100	79·1	20·0
13	E.A.	'Peak 2' fraction from human gastric juice	—	100	79·1	70·9
14	E.A.	'Peak 2' fraction from pig pyloric secretion	0·31	20	79·1	50·1
15	W.O'C.	'Peak 2' fraction from human gastric juice II	—	100	79	47·0
16	W.O'C.	Material not precipitated by Rivanol	0·22	52·5	79	70·9
17	F.W.	'Peak 2' fraction from pig pyloric secretion	0·75	30	72·1	39·0
18	F.W.	Material precipitated by Rivanol	1·2	52·5	72·1	29·8
19	H.D.	Concentrate from pig pylorus (Lederle 45774–133)	50	—	95·6	52·9
20	H.D.	'Peak-2' fraction from pig pyloric secretion, heated for 5 sec.	0·6	60	95·6	78·7
21	W.W.	'Peak-2' fraction from pig pyloric secretion, unheated	0·6	60	89·7	50·7

acid/100 ml., whisked in before filtration. The pepsinogen occurring in the filtrate passed through the resin.

Properties of 'peak-2' material

Several batches of partially purified pyloric secretion were pooled and passed once more through resin XE-64, the 'peak-2' fractions being reprecipitated with ammonium sulphate, dialysed and finally freeze-dried and stored at 4° in the dark in an atmosphere of nitrogen. For each 100 ml. of original secretion the final product weighed 1·05 mg. This is the 'peak-2' concentrate referred to in this paper.

When dried on a watch-glass a solution of it left a shiny brittle transparent almost colourless glaze,

Table 2. *Activity of intrinsic-factor preparations in totally gastrectomized rats*

Figures in parentheses, when given, are control values (without intrinsic factor) for the rats concerned. The mean excretion in 43 gastrectomized rats without intrinsic factor was 95·3±8%. Volumes (marked *) in the third column, when given, represent volumes of original secretion equivalent to the material given. 'Peak 2' is pig pyloric secretion 'peak 2'.

Test no.	Preparation	Volume (ml.) for Test 1 or Weight (μg.) for Tests 2–7	Percentage of vitamin B_{12} excreted
1	'Peak 2'		
	Precipitated by 20% (v/v) of ethanol	1*	81·7 (104·4); 83·3
	Precipitated by 20–30% (v/v) of ethanol	1*	88·4 (95·0); 85·2 (96·0); 80·7 (80·5)
	Precipitated by 20–30% (v/v) of ethanol	2*	80·1; 80·0 (93·5)
	Not precipitated by 30% (v/v) of ethanol	1*	82·3 (96·2); 90·3
2	'Peak 2'	28	64·1 (89·3); 74·5 (80·5); 60·6 (100·0)
	'Peak 2'	1	79·3 (101·1); 77·4 (98·0)
3	'Peak 2' another preparation	10·5	81·8 (93·5); 65·0 (95·0); 75·2 (104·4); 79·3 (96·0); 89·2 (80·5)
	Precipitate of 'peak 2' after heating for 5 sec. in a boiling-water bath	Portion	103·7; 100·8; 94·5; 94·7 (89·3); 96·1
	Supernatant of 'peak 2' after heating for 5 sec. in a boiling-water bath	Portion	103·7; 100·8; 94·5; 94·7 (89·3); 96·1
4	Fractions of Fig. 2		
	Fraction I	20	87·2; 99·9 (80·5)
	Fraction II	20	101·6 (97·0); 94·0
	Fraction III	20	89·0 (95·0); 90·1 (96·2)
	Fraction IV	20	91·4 (101·1); 100·5 (104·4)
	Fraction V	20	83·9; 82·6 (96·0)
	Fraction VI	2	81·1; 96·0 (98·0)
		10	69·0 (92·6)
		20	76·1; 98·0; 79·1 (97·8)
		38	83·6
	Fraction VII	20	87·4; 88·5
	Pooled fractions I–VII	20	79·0; 89·5 (97·0)
5	Concentrate from pig pylorus (Lederle)	500	95·3; 92·1 (104·4); 96·6 (101·1); 93·2; 92·4; 100·2
		250	90·3 (92·6); 98·7 (98·9); 104·4 (99·4)
6	Fractions from 5 (see Table 4)		
	110–115	0·61	99·1 (96·2); 105·1 (97·0)
		0·91	102·5 (97·8); 97·8
	116–134	0·35	67·5 (95·0); 72·7 (101·0); 70·0 (100·0)
	135–144	1·10	93·3 (80·5); 103·0 (96·0)
		1·65	92·0 (89·0); 96·0 (104·2)
	145–149	1·52	106·0 (98·0); 104·5
		2·28	95·0; 93·0 (89·3)
7	'Peak 2' from normal human gastric juice	0·58	87·0 (102·2); 84·4 (85·0); 87·7 (98·0); 83·0 (101·1)
		1·15	77·5 (80·5); 96·2 (99·5); 93·3 (89·3); 99·7 (100·0)
		2·3	89·0 (80·5); 101·2 (99·5); 102·0 (89·3); 100·2 (100·0)
		11·3	79·1 (100·0); 76·3 (96·2); 72·8 (93·3); 80·1; 79·8 (86·5); 78·8 (80·0); 78·0 (80·5); 78·2
		56·5	100·0; 98·0; 100·0; 101·7; 81·8

which was not markedly hygroscopic and dissolved readily in water. The solution, which was neither mucoid nor very viscous, gave no precipitate with 'dialysed iron' or with a final concentration of 10% (w/v) of trichloroacetic acid or 0·6% of perchloric acid. When mixed with cyanocobalamin, no precipitate was formed. A heavy, greyish, waxy precipitate formed with 1% phosphotungstic acid, and an abundant, yellow precipitate with a small volume of 25% (w/v) phosphomolybdic acid in 6·5N-hydrochloric acid. With calcium picrate it gave an amorphous yellow precipitate at neutral pH. A spot of solution dried on Oxoid membrane gave a strong periodic acid–Schiff reaction. Membrane electrophoresis (see below) suggested that the material was far from homogeneous.

Heat coagulability of 'peak-2' concentrate. A neutral, 1% solution of the concentrate sealed in thin-walled glass capillaries was heated in a water bath at various temperatures and for varying lengths of time. Immersion in a boiling-water bath for 5 sec. precipitated about 30% of the material; when this was centrifuged and the supernatant again heated no further precipitate formed, however long the heating. The 5 sec. immersion in boiling water appeared to destroy most (20, 21, Table 1) or all (3, Table 2) of the activity.

Ethanol fractionation of 'peak-2' concentrate. Fractionation of a 1% solution with ethanol at $-10°$ to $-2°$ showed that 27% of the dry weight was precipitated by a final concentration of 20% (v/v) of ethanol, and a further 32·5% on increasing the ethanol concentration to 30% (v/v). These two fractions and the 40·5% not precipitated were tested in gastrectomized rats (1, Table 2). All three were slightly active and ethanol fractionation at this stage did not appear to be a fruitful purification step.

Fractionation with Rivanol. Steinbuch & Quentin (1959) stated that Rivanol (2:5-diamino-7-ethoxyacridine) preferentially precipitates some proteins under carefully controlled pH conditions. When a 1% solution of the 'peak-2' concentrate mentioned above was mixed with 0·2% of Rivanol adjusted to pH 8·0 only a faint turbidity appeared. Cautious addition of alkali caused a marked turbidity at pH 8·4 and a definite precipitate at about pH 8·8. Further alkali caused more precipitate, but there was the risk that Rivanol itself, or rather the free base, might be precipitated as well, as the pH was raised. Since the pH was so critical and at the same time difficult to control in the small volume of unbuffered solution used (0·4 ml.) this method would not be easy to reproduce. Two successive precipitates (together 60–70% of the total) and a non-precipitated portion (30–40% of the total) which were obtained in one fractionation were freed from Rivanol by dialysis and tested. The combined precipitates appeared strongly active, but the non-precipitated material was not (18, 16, Table 1).

In a second Rivanol fractionation about 90% of the starting material was precipitated and was not tested.

Attempts to resolve 'peak 2'. Slight as is the difference between the buffers A and B of Latner *et al.* (1956), it seems quite clear that the first does not, and the second does, easily, elute intrinsic-factor activity from a column of Amberlite XE-64 resin. Their pH is almost identical (A, 4·92; B, 4·96; see below), but that a high ionic concentration itself is not responsible for elution is shown by the fact that buffer A containing 2M-sodium chloride does not elute activity (10, Table 1); however, this may well be because the sodium chloride lowers the pH of buffer A considerably. At the pH level used the resin is only slightly ionized.

The first empirical attempt to resolve 'peak 2' (from pig secretion) was by gradient elution from buffer A to buffer B. The peak was spread, but no significant resolution of material absorbing at 275 mμ was obtained, and no tests for intrinsic-factor activity were made. Later, when a provisional rat assay became available, an attempt was made with buffers intermediate between A and B, as well as A and B, with the results shown in Fig. 2. The composition and pH, measured according to British Standards Specification 1647 (1950), of buffers carefully made up with boiled water is given in Table 3. It will be noted that buffer A 2 is

Table 3. *Composition and* pH *of buffers used for elution*

Buffer A 2 is buffer B diluted 1 in 5. Note marked effect of dilution on pH. The phosphate was in the form of sodium dihydrogen phosphate dihydrate, recrystallized twice.

Buffer	Molarity of phosphate	Vol. of N-NaOH added/l. (ml.)	pH
A of Latner, Merrills & Raine (1956)	0·2	3·0	4·92
A 1	0·2	10·0	5·32
A 2	0·2	14·0	5·50
B of Latner *et al.* (1956)	1·0	70·0	4·96
B diluted 1 in 2	—	—	Approx. 5·26
B diluted 1 in 10	—	—	Approx. 5·64

buffer B diluted 1 in 5, and that the pH of buffer B differs considerably from the 5·2 reported by Latner *et al.* (1956). This discrepancy, and the marked effect of dilution (Table 3), is perhaps less surprising in view of the fact that the solutions concerned are almost outside the buffering range.

Fractions I–VII (Fig. 2) were separately precipitated by full saturation with ammonium sulphate and were exhaustively dialysed. Their total dry weight might be expected to be roughly the same as if buffer B had been used for elution instead of the sequence A1, A2 and B. The total weight, 27·34 mg., i.e. 2·24 mg./100 ml. of original secretion, is only a little higher than usually obtained for 'peak 2', as eluted by buffer B. However, only fraction VI has unequivocal activity in gastrectomized rats (4, Table 2), and if all the activity were to reside in this fraction the dry weight would be equivalent to only 0·18 mg./100 ml. of original secretion. Unfortunately it has not been possible to make more tests on this material.

A similar fractionation with a crude mucosal extract is described in the next section.

Behaviour towards resin chromatography of human gastric juice. When dialysed human gastric juice was passed through a column of resin by the procedure of Latner *et al.* (1956) without prior concentration, the 'peak 2' finally obtained was active both in a pernicious-anaemia patient (15, Table 1) and in gastrectomized rats (7, Table 2), though only, in the rats, at certain dose levels. The dry weight of this preparation was not known, but that of a similar one was 1·85 mg./100 ml. of original juice.

An earlier resin fractionation of an ammonium sulphate precipitate of human gastric juice had given an inactive 'peak 2' (13, Table 1), perhaps because the strongly acid juice had not been neutralized before precipitation.

Behaviour towards resin chromatography of crude concentrate from pig pylorus. The crude concentrate from pig pylorus (Lederle) was shaken with 0·02 M-phosphate buffer, pH 6·8, for 2–3 hr., a few drops of 0·2 N-NaOH being added from time to time to maintain the pH. The faintly turbid solution was centrifuged, dialysed, centrifuged again and the supernatant made up to the ionic composition of buffer A and passed through the resin in the usual way. The column was then eluted with buffers A, A1, A2 and B in turn, and the absorption, at 275 mμ, of the fractions was measured. The rate of flow was roughly constant, but the fractions were collected at different time intervals and therefore

Table 4. *Condensed summary of fractionation of 1·4 g. of pig pylorus concentrate (Lederle) on Amberlite resin* XE-64

For composition of buffers, see Table 3. Rate of flow was roughly constant but fractions were collected at different time intervals and therefore varied in volume. For assays, see 6, Table 2.

Fractions	Buffer	Volume (ml.)	Dry wt. (mg.)	Intrinsic-factor activity in gastrectomized rats
1–14	A	1182	—	Not tested
15–61	A1	186	2·4	Not tested
62–90	A1	461	41·6	Not tested
91–109	A1	352	11·6	Not tested
110–115	A1	74	1·69	Inactive
116–134	A2	58	0·97	Active
135–144	A2	110	3·07	Inactive
145–149	B	88	4·2	Inactive

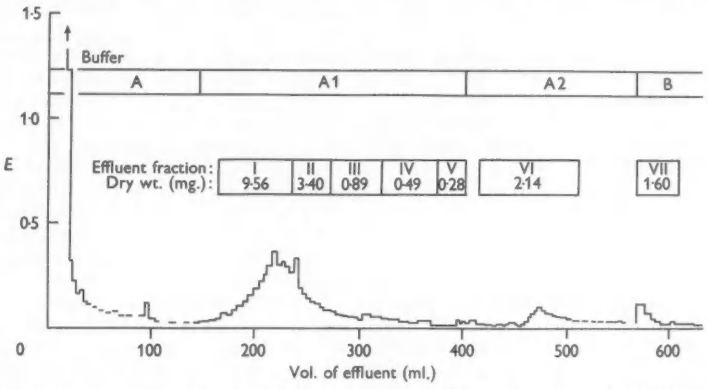

Fig. 2. Absorption at 275 mμ (1 cm. path) of successive eluates from a column of Amberlite resin XE-64 loaded with a concentrate derived from 1220 ml. of pig pyloric secretion. For composition of buffers A, A1, A2 and B see Table 3. Eluates were pooled in fractions I–VII; the dry weights were those of the dialysed ammonium sulphate precipitates.

varied considerably in volume. Table 4, giving the results in condensed form, and 6, Table 2, shows that only the first part of the A2 eluate was active in gastrectomized rats. The dry weight of this fraction was 0·97 mg., and as it is derived from about 1·4 g. of the crude Lederle powder, the purification would be nearly 1500-fold, if all the activity had collected in this one fraction. Further, it would appear that had only buffers A and B been used as recommended by Latner *et al.* (1956), the dry weight of the active 'peak 2' would have been nearly 70 times as great.

The dose of the active fraction given to the gastrectomized rats was 0·35 μg., which would have been equivalent to 0·5 mg. of the original powder. Yet the crude powder had been shown to be inactive in gastrectomized rats at this dose and at half this dose (5, Table 2). Some inhibitory factor or factors have possibly been removed, and this has been confirmed by M. A. Sheikh & K. B. Taylor (unpublished work).

Membrane electrophoresis. Various fractions were tested in 0·1 M-veronal–acetate buffer (Oxoid), pH 8·6, and/or in 0·1 M-phosphate buffer, pH 6·8 or 7·0. Half-neutralized 0·1 M-acetic acid was also used in a few cases.

On electrophoresis a sample of 'peak 1' gave several distinct nigrosine-staining spots, suggesting that the electrophoresis technique was adequate. However, 'peak 2' preparations, apart from a spot moving towards the cathode (at pH 6·8 or 8·6), gave an indistinct blur spreading in both directions from the point of application. The cathodal spot is not associated with intrinsic-factor activity since, of fractions I–VII of Fig. 2, only the inactive fraction VII contained this component. The membrane-electrophoretic pattern of VI (which was active) was indistinguishable from those of I–V (which were inactive), and the technique has so far been disappointing. The cathodal spot might, however, serve as a marker during elution from Amberlite XE-64 if its appearance immediately after the active fraction can be confirmed under other conditions. It is fully realized that intrinsic factor itself may not stain with nigrosine.

DISCUSSION

The fact that the three different starting materials (pig pyloric secretion, a concentrate from pig pylorus and normal human gastric juice) behave similarly on chromatography on Amberlite resin XE-64, suggests that this purification procedure, described by Latner *et al.* (1956), is a reliable and reproducible one. By choosing buffers graded more finely than their buffers A and B a product many times more active than their 'peak 2' can be obtained. Thus if the recovery in the A–A1–A2–B procedure were to be the same as in the simple A–B system, a 12-fold purification would have been obtained with the pyloric secretion, and one of 70-fold with the mucosal concentrate. It is possible that choice of slightly different buffers for elution might give still further purification, and washing of the column with buffer A containing 2 M-NaCl (which does not remove intrinsic factor) might also be worth testing. A technical point needing investigation is whether intrinsic-factor activity would eventually be eluted by buffer A1 if a sufficient volume were used, for this could lead to poor recovery. In the two runs reported here buffer A1 has not removed detectable intrinsic factor, nor has much tailing occurred in the elution with A2, another possible cause of low recovery. Whether the slight activity observed in the single test of 'peak 1' (8, Table 1) is genuine has not been investigated.

Measurement of the ultraviolet absorption, which is a useful guide to the course of elution when buffers A and B only are used, is of little or no value in the more refined elution process described here, and a blind application of the process would seem to be the only course available at present, with the possible exception that a nigrosine-staining spot, which moves fast towards the cathode on membrane electrophoresis, has so far emerged from the Amberlite column just after the active material and may have limited value as a marker. In this connexion Jacob, Williams, Howe & Glass (1959), purifying pig intrinsic factor by the totally different procedures of high-speed centrifuging and zone electrophoresis, suggest (with marked reservation) that intrinsic factor may be associated with a cathodal component. It would be interesting to see whether chromatography of their product on Amberlite XE-64 would separate intrinsic factor from their cathodal component. It would appear that their purest product is of much the same activity as the 'peak 2' concentrate obtained here by the procedure of Latner *et al.* (1956). Fraction VI of Fig. 2 and 116–134 of Table 4 should be several times more active, though the opportunity has not occurred of testing these directly on a pernicious-anaemia patient.

Pyloric secretion would not appear to be a more favourable starting point for purification of intrinsic factor than a mucosal extract, which is, of course, very much easier to obtain.

Membrane electrophoresis still indicates that the purest products are grossly impure, yet the 'peak-2' concentrate is active at 0·3 mg. in pernicious-anaemia patients (14, Table 1), and the purer products should be several times as active.

A difficulty which has always beset work on intrinsic factor is the, at best, semi-quantitative nature of the assays. Should the use of gastrecto-

mized rats for assay be possible, the easy replication of tests would go some way to meet this difficulty. However, the hope of an accurate assay will be somewhat dashed by (*a*) the presence in partly purified intrinsic-factor preparations of unknown amounts of inhibitor or inhibitors, (*b*) the marked dependence, in some cases, of activity on the size of the dose (e.g. 7, Table 2), (*c*) the possibility of quantitative differences in response of a test animal to intrinsic factor (and inhibitors) from a different animal species and (*d*) the possibility of a test animal becoming refractory. These points are discussed more fully by M. A. Sheikh & K. B. Taylor (unpublished work).

SUMMARY

1. Preliminary attempts to purify and examine the properties of intrinsic factor are reported.

2. Fractionation by chromatography on an exchange resin, as described by Latner, Merrills & Raine (1956) for a concentrate from pig gastric mucosa, gives closely similar results with pig pyloric secretion and human gastric juice, and appears to be a reliable and reproducible method.

3. The active fraction can be obtained several times purer by modifications of the exchange-resin process. There are reasons for thinking that the final product might be active in pernicious-anaemia patients in a dose of about 30 μg.

4. Membrane electrophoresis suggests that the purest product is still far from homogeneous.

We are grateful to Professor Sir Howard Florey and Professor L. J. Witts for suggesting this collaboration and for their continued interest; to Mr J. Kent for tending the pig and collecting the pyloric secretion; to Miss B. Mallett for measurement of radioactivity of some of the human material; and to Mrs S. Groves for technical assistance.

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Synthesis of the α- and γ-Isomers of Glutamylcystinylvaline

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It has been shown (Arnstein & Morris, 1959, 1960*b*) that L-cystinyl-L-valine is available for penicillin biosynthesis in washed mycelium of *Penicillium chrysogenum* by a pathway other than that involving breakdown to free amino acids. Following this observation, the mycelium of *P. chrysogenum* was examined for the presence of cystinylvaline or of similar peptides containing both cystine and valine; at an early stage in this work there was some evidence of the presence of glutamylcystinylvaline in mycelial extracts. In order to assist in the identification of this peptide the α- and γ-isomers of glutamylcystinylvaline were synthesized.

The main problem in the synthesis of glutamyl peptides is that of producing, unequivocally, either peptides in which the α-carbonyl group is a member of the peptide chain, i.e. with part structure (A), or those in which the γ-carbonyl group participates in peptide-bond formation, as shown in part structure (B) below. Amiard, Heymès & Velluz (1956) have reported the synthesis of γ-glutamylcysteinylglycine (glutathione), in which the desired γ-linkage of glutamic acid to the rest of the molecule was

(A) (B)

obtained by the use of the triphenylmethyl (trityl) *N*-terminal-protecting group, the latter preventing by steric hindrance the participation of the α-carboxyl group in peptide-bond formation. In view of the similarity between glutathione and γ-glutamylcystinylvaline, the trityl-protecting group was used in the synthesis of this γ-peptide.

Trityl-L-glutamic acid was coupled with *S*-benzyl-L-cysteinyl-L-valine ethyl ester, prepared from the benzyloxycarbonyl-*S*-benzyl dipeptide ester (Arnstein & Morris, 1960*a*) by the action of hydrogen bromide in acetic acid, the di*cyclo*hexylcarbodi-imide method of Sheehan & Hess (1955) being used. The fully protected tripeptide ester was saponified with ethanolic potassium hydroxide to give the trityl peptide in 77% yield. The trityl group was rather labile and was lost to a considerable extent during the purification procedure at this stage. All the trityl derivatives used in this synthesis gave ninhydrin-positive spots on paper chromatograms, the trityl group presumably being lost during the heating of the paper.

Complete removal of the trityl group was achieved by heating the protected peptide in 50% (by vol.) acetic acid at 100° for a short time. The chromatographically pure *S*-benzyl tripeptide was reduced with sodium in liquid ammonia to glutamylcysteinylvaline, which was not isolated but oxidized directly to the disulphide form. Chromatography in solvent *A* (see Experimental section) revealed the presence of two components, one of which was present in only a small amount. The R_F of the minor component was the same as that of synthetic α-peptide. A portion of the mixture was oxidized with performic acid to the cysteic acid (SO_3H) form and subjected to paper electrophoresis. At pH 2 the mixture behaved as one component. At pH 3, however, where the free α-carboxyl group of the γ-peptide would be charged to a greater extent than the corresponding free γ-carboxyl group of the α-peptide, the major component migrated much faster than did the minor one. From the above-mentioned evidence it was concluded that the peptide was predominantly the required γ-isomer but contained a small proportion of the α-form. It appears therefore that steric shielding of the α-carboxyl group during peptide-bond formation was not complete. The pure γ-peptide was obtained by elution of the required band (R_F 0·12 in solvent *A*) from preparative-scale chromatograms).

The dehydration of toluene-*p*-sulphonyl (tosyl)-glutamic acid to tosylpyroglutamic acid has been known for some time (Harington & Moggridge, 1940) but the application of this reaction to the synthesis of α-glutamyl peptides has been relatively recent. Rudinger (1954*a*, *b*) has reported the selective synthesis of several α-glutamyl peptides by coupling the acid chloride derivative of tosylpyroglutamic acid with either amino acids or their esters. Accordingly, this method was used to prepare α-L-glutamyl-L-cystinyl-L-valine.

In a preliminary experiment tosyl-L-pyroglutamyl chloride (Rudinger, 1954*a*), prepared by refluxing tosyl-L-glutamic acid with thionyl chloride, was coupled with *S*-benzyl-L-cysteinyl-L-valine ethyl ester to give a very poor yield of material which could not be purified. Coupling of the *S*-benzyl dipeptide ester with tosyl-L-pyroglutamic acid by the di*cyclo*hexylcarbodi-imide method was successful, however, the required tosyl tripeptide ester being obtained in good yield. Treatment of this product with ethanolic potassium hydroxide gave a high yield of the saponified peptide. A small portion of this material was converted into the *cyclo*hexylamine salt; this derivative of saponified peptide intermediates has been found very useful in obtaining crystalline material from these compounds, which are otherwise often very difficult to crystallize (Arnstein & Morris, 1960*a*). Removal of the tosyl- and *S*-benzyl-protecting groups with sodium in liquid ammonia proceeded smoothly, the use of IRC-50 cation-exchange resin as described by Rudinger (1954*b*) facilitating the removal of inorganic ions from the product. Once again chromatography revealed the presence of two spots of the same R_F values as described for the γ-peptide, only with the orders of magnitude reversed. The major spot was identified as the α-peptide by electrophoresis of the $-SO_3H$ form at pH 3·5. The pure α-peptide was separated from the small amount of contaminating γ-material by crystallization from aqueous ethanol. It would appear that the pyrrolidone method of synthesis of α-glutamyl peptides is not completely specific. Rudinger & Czurbová (1954) have reported that the pyrrolidone ring can be opened with amino acid (and, presumably, peptide) esters, the reaction, however, being slow. It seems probable therefore that concomitant attack of *S*-benzyl-L-cysteinyl-L-valine ethyl ester on the pyrrolidone ring took place during the coupling reaction, giving rise to some γ-peptide.

Both α- and γ-peptides were obtained as dihydrates which could not be converted into the anhydrous forms by drying at above 100°. This tendency of peptides containing cystine and valine to form hydrates has been found with isomers of cystinylvaline (Arnstein & Morris, 1960*a*).

EXPERIMENTAL

Paper chromatography. All R_F values refer to descending chromatograms on Whatman no. 1 paper unless otherwise stated. The solvent systems used had the following compositions (by vol.): *A*, butanol–acetic acid–water, 63:10:27;

B, amyl alcohol–pyridine–water, 7:7:6; *C*, phenol–water, 5:2 (with NH_3 in the tank).

Electrophoresis. Electrophoresis was carried out on Whatman 3MM paper with either an uncooled apparatus (for voltages up to 1 kv) or a special water-cooled apparatus for high voltages. The pyridine–acetic acid buffer was made by diluting a mixture of acetic acid (100 ml.) and pyridine (10 ml.) to 1 l. with water, giving a stock solution of 1·75 M-buffer, pH 3·5. The stated buffer concentration refers to that of the anionic component and was obtained by suitable dilution of the stock solution.

Synthesis of peptides. The routes to the γ- and α-tripeptides are outlined in Figs. 1 and 2 respectively. All melting points are uncorrected.

Synthesis of γ-L-glutamyl-L-cystinyl-L-valine

Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-valine ethyl ester (I, 6 g.; Arnstein & Morris, 1960*a*) was dissolved in dry acetic acid (100 g.) containing HBr (20 g.) and allowed to stand at room temperature for 1 hr. An equal volume of acetic acid was added and the diluted solution was freeze-dried. The crude product was dissolved in ethyl acetate (10 ml.) and crystallized by addition of light petroleum (b.p. 60–80°) to give a product (5·2 g., m.p. 125–135°) which contained some *S*-benzylcysteine (shown by chromatography in solvent *A*). The impure material was dissolved in ethyl acetate (200 ml.), extracted with aqueous Na_2CO_3 (10%, w/v; 2 × 15 ml. portions) and then washed with water until the washings were neutral. After drying (anhydrous Na_2SO_4) for 1 hr., ethanolic N-HCl (15 ml.) was added and the solution was evaporated to dryness *in vacuo*; the resulting oil was crystallized from ethyl acetate–light petroleum (b.p. 60–80°) to give 3·65 g. (77%) of product, m.p. 121–122°. For analysis a small portion was recrystallized as before to give *S-benzyl-L-cysteinyl-L-valine ethyl ester hydrochloride* (II), m.p. 123–125° (Found: C, 54·2; H, 7·42; N, 7·23. $C_{17}H_{27}O_3N_2S\cdot Cl$ requires C, 53·8; H, 7·19; N, 7·47%).

Triphenylmethyl (trityl)-L-glutamic acid triethylamine salt (III, 4·6 g.), prepared by the method of Amiard *et al.* (1956), and *S*-benzyl-L-cysteinyl-L-valine ethyl ester hydrochloride (II, 3·5 g., 1 mol.prop.) were dissolved together in dry ethylene dichloride (50 ml.). Di*cyclo*hexylcarbodi-imide (2·12 g., 1·1 mol.prop.) was added, the mixture was shaken mechanically for 2 hr. and then left at room temperature overnight. The precipitated di*cyclo*hexylurea was filtered off and the solvent was evaporated *in vacuo* to leave a residue which was dissolved in ethyl acetate (150 ml.) and extracted with N-HCl (3 × 20 ml. portions) and water (2 × 25 ml. portions) respectively. Benzene was added and the solvent was evaporated *in vacuo*. The residue was dissolved in dry ethyl acetate and was crystallized by addition of light petroleum (b.p. 60–80°) to give crude product (6·3 g., 85%), m.p. 85–95°. Electrophoresis in 2 M-acetic acid on Whatman 3MM paper indicated that the product contained some residual tritylglutamic acid. The latter was removed by 10 successive extractions of an ethyl acetate solution of the product (387 mg.) with 0·01 N-$NaHCO_3$ (10 × 5 ml. portions, 1 mol.prop.) to give *triphenylmethyl-γ-L-glutamyl-S-benzyl-*

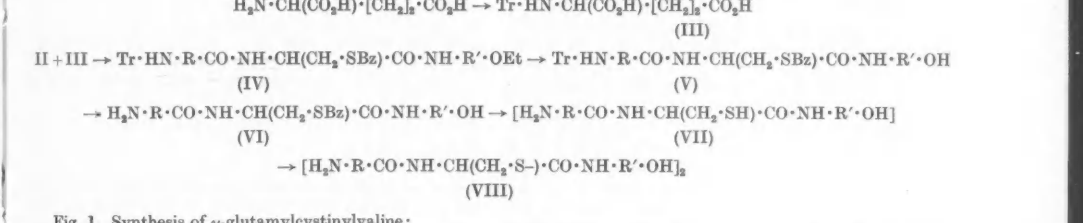

Fig. 1. Synthesis of γ-glutamylcystinylvaline:
Z, $C_6H_5\cdot CH_2\cdot O\cdot CO$; Bz, $CH_2\cdot C_6H_5$; Tr, $(C_6H_5)_3\cdot C$; Et, C_2H_5; R, $CH(CO_2H)\cdot[CH_2]_2$; R′, $CH(CH\cdot Me_2)\cdot CO$.

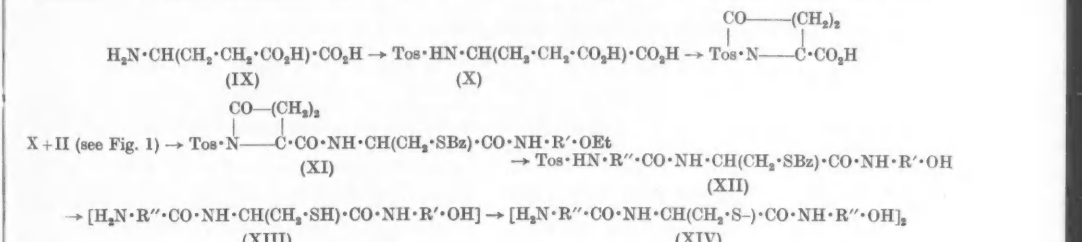

Fig. 2. Synthesis of α-glutamylcystinylvaline:
Tos, $C_6H_4\cdot CH_3\cdot SO_2$; Et, C_2H_5; Bz, $CH_2\cdot C_6H_5$; R′, $CH(CH\cdot Me_2)\cdot CO$; R″, $CH(CH_2\cdot CH_2\cdot CO_2H)$.

L-*cysteinyl*-L-*valine ethyl ester* (IV), 220 mg. (54%), m.p. 112–114°, $[\alpha]_D^{21}$ +15·4° (*c*, 1·04 in ethyl acetate) (Found: C, 69·8; H, 6·71; N, 6·30. $C_{41}H_{47}O_6N_3S$ requires C, 69·5; H, 6·6; N, 5·93%).

The fully protected tripeptide (IV; 1·7 g.) was dissolved in ethanolic N-KOH (12 ml.) and allowed to stand at room temperature for 40 min.; the solution was then brought to pH 7 with N-HCl and, after dilution with water, $NaHCO_3$ (2%, w/v) was added to bring the pH to 8·5. The alkaline solution was extracted with ethyl acetate (3 × 10 ml. portions) and the latter was washed with $NaHCO_3$ (2%); the combined aqueous fractions were acidified to pH 2 with conc. HCl and extracted with ethyl acetate (3 × 10 ml. portions). After drying (anhydrous Na_2SO_4) the solvent was removed to give 1·5 g. (93%) of crude product (V), m.p. 125–130°, with R_F 0·63 in solvent *B*. Several recrystallizations from ethyl acetate–light petroleum (b.p. 80–100°) gave a product, m.p. 145–150°, which could not be purified further and which was shown by chromatography in solvent *B* to contain some detritylated material (VI), R_F 0·20. The product (V, 2 g.) was suspended in water (20 ml.) at 100° and acetic acid (20 ml.) was added; starting material dissolved immediately and a product was precipitated. Water (40 ml.) was added and the reaction mixture was freeze-dried to give 1·3 g. (100%) of γ-L-glutamyl-*S*-benzyl-L-cysteinyl-L-valine (VI), m.p. 170–175°. This product was chromatographically pure with R_F values 0·60 in solvent *A* and 0·20 in solvent *B*; it was used without further purification.

The *S*-benzyl peptide (VI, 880 mg.) was dissolved in dry liquid NH_3 (100 ml.) contained in a three-necked flask fitted with a mechanical stirrer and a $CaCl_2$ guard tube. Sodium was introduced in small portions, with stirring, until a permanent (7 min.) blue colour was obtained; 250 mg. of sodium was required. Ammonium acetate (3 g.) was added, the NH_3 was evaporated as rapidly as possible and the residue was placed in a desiccator over conc. H_2SO_4 overnight. The dry crude product was dissolved in ice–water (100 ml.) and was stirred for 30 min. at 10° with IRC-50 in the NH_4^+ form (10 g., wet wt.; The British Drug Houses Ltd., London). The resin was filtered off and the filtrate was freeze-dried. The residue was taken up in water (20 ml.), the pH was adjusted to 8·5 with 2N-NH_3 soln., a trace of $FeCl_3$ was added and O_2 was bubbled through the solution [to oxidize the thiol form of the peptide (VII) to the disulphide form (VIII)] until the blue colour was discharged; the solution then gave a negative test for thiols with sodium nitroprusside. Freeze-drying of the solution gave crude product (700 mg.) which was crystallized from aqueous ethanol to give a product (600 mg., 92%) which gave two spots, R_F 0·12 (major) and R_F 0·16 (minor) in solvent *A*. The faster-moving material was later identified (see below) as α-L-glutamyl-L-cystinyl-L-valine. For purification the mixture (250 mg.) was chromatographed on Whatman 3MM paper (four sheets) in solvent *A* for 45 hr. The γ-peptide band (R_F 0·12) was eluted with 0·3M-NH_3 soln. (200 ml.) and the eluate was concentrated *in vacuo* and desalted on Zeo-Karb 215 (The Permutit Co. Ltd., London). The peptide was displaced from the column with 0·3M-NH_3 soln. and the eluate was concentrated *in vacuo* to 3 ml. Addition of ethanol and ether gave a solid product, which, after drying *in vacuo* for 4 hr. at 100° and then 2 hr. at 110°, gave analytically pure γ-L-*glutamyl*-L-*cystinyl*-L-*valine dihydrate* (VIII), m.p. > 300°, $[\alpha]_D^{23}$ −17·7° (*c*, 0·99 in 5N-HCl) (Found: C, 42·4; H, 6·51; N, 11·5. $C_{26}H_{44}O_{12}N_6S_2,2H_2O$ requires C, 42·6; H, 6·6; N, 11·4%).

Synthesis of α-L-*glutamyl*-L-*cystinyl*-L-*valine*

Toluene-*p*-sulphonyl (tosyl) L-glutamic acid (IX, 10 g.), prepared by the method of Rudinger (1954*a*) was converted into tosyl-L-pyrrolid-5-one-2-carboxylic acid (X, 6·1 g., 75%), m.p. 129–130°, identical with the material described by Rudinger (1954*a*).

The above product (X, 2·65 g.) and *S*-benzyl-L-cysteinyl-L-valine ethyl ester hydrochloride (II, 3·5 g., 1 mol.prop.) were dissolved together in dry ethylene dichloride (40 ml.). Triethylamine (0·94 g., 1 mol.prop.) and di*cyclo*hexylcarbodi-imide (2·14 g., 1·1 mol.prop.) were added to this solution; considerable evolution of heat, accompanied by deposition of di*cyclo*hexylurea, occurred, and the reaction mixture was cooled rapidly to room temperature before being shaken mechanically for 4 hr. The urea was filtered off and the solvent was removed *in vacuo*. The residue was dissolved in $CHCl_3$ (100 ml.) and was washed successively with water, N-HCl, water, 50% sat. $NaHCO_3$ and water. The $CHCl_3$ was then evaporated *in vacuo* and the residue was taken up in ethyl acetate (more insoluble di*cyclo*hexylurea was removed by filtration); slow addition of light petroleum (b.p. 80–100°) induced crystallization and, after standing overnight at −5°, the crude product (4·26 g., 75%), m.p. 92–95°, was obtained. Recrystallization of a small quantity from ethyl acetate–light petroleum (b.p. 60–80°) gave analytically pure *toluene*-p-*sulphonyl*-L-*pyrrolidone*-S-*benzyl*-L-*cysteinyl*-L-*valine ethyl ester* (XI), m.p. 96–100° (Found: C, 57·8; H, 6·5; N, 6·6. $C_{29}H_{37}O_7N_3S_2$ requires C, 57·7; H, 6·1; N, 6·96%). The R_F in solvent *B* was 0·93.

The fully protected peptide ester (XI, 2·01 g.) was treated with ethanolic 2N-KOH (8·34 ml.) for 15 min. at room temperature. The reaction mixture was then poured into 50% sat. $NaHCO_3$ (100 ml.) and extracted with ethyl acetate (3 × 20 ml.). The aqueous phase was acidified to Congo red with N-HCl and extracted again with ethyl acetate (3 × 20 ml.); addition of light petroleum (b.p. 60–80°) gave chromatographically pure product (1·70 g., 85%), R_F 0·61 in solvent *B*. A small quantity was recrystallized from ethyl acetate alone to give *toluene*-p-*sulphonyl*-α-L-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*valine* (XII), m.p. 186–188° (Found: C, 54·5; H, 6·26; N, 6·94. $C_{27}H_{35}O_8N_3S_2$ requires C, 54·7; H, 5·9; N, 7·1%). A portion was dissolved in ethyl acetate, and *cyclo*hexylamine and ether were added to give the tosyl peptide di*cyclo*hexylamine *salt*, m.p. 180° (Found: N, 8·78. $C_{39}H_{61}O_8N_5S_2$ requires N, 8·86%).

The tosyl peptide (XII, 750 mg.) was reduced with sodium in liquid NH_3 under the same conditions as those described for the γ-peptide; 270 mg. of sodium was needed. After oxidation of the peptide from the thiol (XIII) to the disulphide (XIV) form, the aqueous solution was freed from SO_4^{2-} and SO_3^{2-} ions by the method of Rudinger (1954*b*), M-barium acetate being added until no further precipitate was formed. The solution was filtered, the precipitate was washed on the filter with water and the combined filtrate and washings were concentrated *in vacuo* to 30 ml. This solution was applied to a column of IRC-50 (40 g., wet wt., in the NH_4^+ form) and eluted with water until a total volume of 240 ml. was obtained; the latter

Table 1. *Summary of the chromatographic and electrophoretic properties of the α- and γ-peptides*

—, Signifies not determined.

	Chromatography*			
	R_F (disulphide form)		R_F (–SO_3H form)	
Solvent	α-Peptide	γ-Peptide	α-Peptide	γ-Peptide
A	0·16	0·12	—	—
B	—	—	0·43	0·48
C	0·21	0·42	0·17	0·16

	Electrophoresis† of –SO_3H forms		
		Migration (cm.)	
Conditions	Time	α-Peptide	γ-Peptide
2 N-Acetic acid, pH 2·1; 25 v/cm./26 mA	4 hr.	3·0	3·0
0·35 M-Pyridine–acetic acid, pH 3·5; 19 v/cm./14 mA	1 hr.	9·0	13·0
0·35 M-Pyridine–acetic acid, pH 3·5; 66 v/cm./24 mA	20 min.	2·3	5·6

* Whatman no. 1 paper. † Whatman 3MM paper.

was concentrated *in vacuo* and freeze-dried to give crude α-L-glutamyl-L-cystinyl-L-valine (XIV, 600 mg.). Chromatography in solvent *A* revealed the presence of two main spots, R_F 0·16 (major) and R_F 0·11 (minor), the latter being identical with that of γ-peptide marker; very small traces of other spots were also found. Chromatography in solvent *C* gave a similar picture: major spot, R_F 0·21; minor spot, R_F 0·42 (γ-peptide marker gave R_F 0·41). The crude peptide (530 mg.) was crystallized from aqueous ethanol to give chromatographically pure material (200 mg., R_F 0·16 in solvent *A*); one further crystallization, followed by drying at 110° for 2 hr., gave analytically pure α-L-*glutamyl*-L-*cystinyl*-L-*valine dihydrate* m.p. > 300°, $[\alpha]_D^{23}$ –12·2° (*c*, 0·98 in 5 N-HCl) (Found: C, 42·9; H, 6·6; N, 11·4. $C_{27}H_{44}O_{12}N_6S_2,2H_2O$ requires C, 42·6; H, 6·6; N, 11·4%).

A summary of the chromatographic and electrophoretic properties of the α- and γ-peptides in both their disulphide and oxidized (–SO_3H) forms is given in Table 1.

SUMMARY

1. The synthesis of the α- and γ-forms of L-glutamyl-L-cystinyl-L-valine is reported.

2. The limitations of the methods used are described.

I should like to thank Dr H. R. V. Arnstein for helpful advice and encouragement, and to express my appreciation to Mr E. J. Toms for skilled technical assistance.

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Sulphur-Containing Amino Acids and Peptides in the Mycelium of *Penicillium chrysogenum*

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Washed mycelium of *Penicillium chrysogenum* is able to utilize L-cystinyl-L-valine for penicillin biosynthesis by a pathway not involving breakdown to the free amino acids (Arnstein & Morris, 1960*b*). It is possible, however, that some further modification of the dipeptide may be necessary before the latter is incorporated into penicillin, and

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therefore the mycelium of *P. chrysogenum* has been examined for the presence of cystinylvaline, or of modified peptides containing both cystine and valine. This paper describes the isolation of such a peptide, containing α-aminoadipic acid. This work has been reported briefly elsewhere (Arnstein, Morris & Toms, 1959).

EXPERIMENTAL

Methods and materials

Preparation of washed mycelium. Fermentation of *P. chrysogenum* WIS 51–20 F3 was carried out as described by Halliday & Arnstein (1956), but in the absence of phenylacetic acid. After 63 hr. the fermentation was stopped and the mycelium was harvested by filtration. The mycelium was washed three times by suspension in 0·01 M-buffer and filtered to give a mycelium of 13% wet wt.; the mycelium was used at once.

Penicillin bioassay. Penicillin was estimated by the cup-plate method with *Bacillus subtilis* (ICI strain N.C.T.C. 8241) as test organism (Humphrey & Lightbown, 1952).

Paper chromatography. Preliminary work indicated the necessity of using washed papers. Whatman 3MM papers were washed successively (by descending solvent flow in a chromatography tank) with M-ammonium acetate, M-acetic acid, water and ion-exchange water. The papers were dried in an oven and checked for removal of peptides and amino acid by chromatography (solvent *B*) of a 0·3 M-NH_3 soln. eluate of a sample strip of paper. The solvent systems used had the following compositions (by vol.): solvent *A*, butanol–acetic acid–water, 63:10:27; solvent *B*, phenol–water, 5:2 (with NH_3 in the tank); solvent *C*, pyridine–water, 4:1. Where necessary, chromatograms were developed with ninhydrin (0·1% in butanol containing 2% of water).

Paper electrophoresis. Washed Whatman 3MM paper was used unless otherwise stated. Pyridine–acetic acid buffers were prepared as follows: (*a*) pH 3·5 buffer, a mixture of acetic acid (100 ml.) and pyridine (10 ml.) diluted to 1 l. with water; (*b*) pH 5 buffer, acetic acid (105 ml.) and pyridine (140 ml.) diluted to 1 l. with water. The quantities mentioned give 1·75 M-buffers, which were diluted appropriately before use. The stated concentration refers to that of the anionic component.

Radioactivity measurements. Radioactivity on paper chromatograms was located and measured on a two-dimensional scanner. Radioactivity of materials in solution was determined, after evaporation of samples on to 2 cm.² polythene disks containing lens tissue, with a thin end-window Geiger–Müller tube. Measurements of radioactivity were made mainly for detection of labelled materials and were not intended to be a basis for strictly quantitative estimation.

Radioactive compounds. Uniformly labelled L-[^{14}C]valine was obtained from The Radiochemical Centre, Amersham, Bucks.

Unlabelled compounds. γ-L-Glutamylcysteicylvaline and cysteicylvaline were obtained by performic acid oxidation of the disulphide form of the peptides (Morris, 1960; Arnstein & Morris, 1960*a*); γ-glutamylcysteicylglycine (GSO_3H) was similarly obtained from glutathione (GSH). ββ-Dimethylcysteic acid (penicillaminic acid) was prepared either by degradation of benzylpenicillin to penicilloic acid, followed by performic acid oxidation, or by oxidation of penicillamine.

Incubation of washed mycelium with L-[^{14}C]*valine.* In order to facilitate the subsequent isolation of metabolic products derived from valine, washed mycelium was incubated with L-[^{14}C]valine for 1 hr. as described below. Portions (10 g. wet wt.) of mycelium (giving 10 units of penicillin/ml./hr.) were added to each of six Erlenmeyer flasks (500 ml.) containing medium (100 ml.) of the following composition: 0·01 M-phosphate buffer, pH 7, glucose (1%, w/v), potassium phenylacetate (0·005%, w/v) and L-[^{14}C]valine (5 mg., 8·3 μc). The flasks were incubated on a rotary shaker (140 rev./min.) for 1 hr. at 24°. After incubation the contents of all six flasks were combined and filtered. The mycelium was washed with 0·01 M-phosphate buffer, pH 7.

Extraction of amino acids and peptides from mycelium

The mycelium obtained above was transferred immediately to boiling aqueous ethanol (70%, v/v; 200 ml.) and boiled for 10 min. The suspension was filtered and the residue on the filter was boiled once more with ethanol (70%; 100 ml.). The combined filtrates were cooled at 0° and centrifuged and the sediment was treated again with ethanol. All the ethanol extracts were combined and concentrated *in vacuo* until all the ethanol had been removed. The aqueous solution was centrifuged and the supernatant solution was applied to a column of Dowex-50 resin (12% cross-linked, 200–400 mesh, Microchemical Specialties Co., Berkeley, Calif., U.S.A.) of dimensions 75 cm. × 1·8 cm. diam. The column was eluted with water until the eluate was neutral and the amino acids and peptides were displaced from the column with 0·3 M-NH_3 soln. (700 ml.). The alkaline eluate was concentrated *in vacuo* below 40° to 250 ml. and then freeze-dried to give the crude desalted amino acid–peptide mixture.

Removal of non-sulphur-containing amino acids and peptides

The amino acid–peptide mixture obtained after the desalting step was treated with performic acid (10 ml. of 98% formic acid + 3 ml. of 100 vol. H_2O_2) at room temp. for 1 hr. to oxidize cystine and cystine-containing peptides to the corresponding sulphonic acids. After addition of water (100 ml.) the reaction mixture was freeze-dried. The residue was dissolved in water (20 ml.) and loaded on to a Dowex-50 column (75 cm. × 1·8 cm. diam.) as before. The acidic sulphur-containing materials were eluted with water until the eluate was neutral. (Measurement of radioactivity in a sample of further eluate showed that the elution was complete.) The eluate was freeze-dried and its radioactivity was determined. This fraction was usually contaminated with a trace of valine, which was, however, completely removed by subsequent chromatography on paper with solvent *B* (see below), as shown in a control experiment with a mixture of labelled valine and γ-glutamylcysteicylvaline.

Fractionation of S*-containing amino acids and peptides*

Samples of the mixture were streaked in bands (usually 30 cm.) on washed Whatman 3MM paper and chromato-

graphed in solvent *B*. Cysteic acid, cysteicylvaline, γ-glutamylcysteicylvaline, GSO_3H and penicillaminic acid were used as markers. After running, the paper was dried and radioactive bands were located by scanning. Non-radioactive bands were detected by ninhydrin staining of thin strips cut from either end of the streaked area. Both active and inactive bands were cut out and eluted with 0·3 M-NH_3 soln.; the rest of the chromatogram was developed with ninhydrin to locate the markers. The eluted bands were then subjected separately to paper electrophoresis at 700 v in 2 N-acetic acid for 6 hr. The various bands were detected as before and the composition of the components was established either by comparison with authentic materials or by hydrolysis. Further purification, when necessary, was achieved by chromatography in solvent *C*, two-dimensional chromatography in solvents *A*–*C*, or by electrophoresis at 66 v/cm. in 0·35 M-pyridine–acetic acid, pH 3·5 or pH 5.

Quantitative estimation of peptide hydrolysates

A sample of peptide, the constituent amino acids of which had been determined previously by hydrolysis, was hydrolysed in 6 N-HCl at 105° for 18 hr. The acid solution was evaporated to dryness twice with water; the residue was subjected to two-dimensional analysis on Whatman no. 3 paper by electrophoresis at 3·5 kv in 0·175 M-pyridine–acetic acid, pH 5, and chromatography in solvent *A*. After the first dimension, quantitative markers of the constituent amino acids, in an appropriate range of concentrations, were applied to the paper. After the second dimension, the concentrations of the peptide-hydrolysate amino acids were determined by comparison with the standard markers, by the method of Mandelstam & Rogers (1959).

RESULTS

Isolation of S*-containing amino acids and peptides*

The crude, desalted, mycelium extract obtained from the first column had a radioactivity of 11·5 μc and weighed 575 mg. After oxidation the sulphur-containing materials weighed 189 mg. and the activity was 0·4 μc.

Fractionation of S*-containing material*

Chromatography. Paper chromatography in solvent *B* resolved the mixture into two ninhydrin-positive bands. Band P (R_F 0·07) was non-radioactive and had the same R_F as both the cysteic acid and GSO_3H markers. Band Q (R_F 0·20) was radioactive (activity was estimated to be 0·14 μc), with the same R_F as both γ-glutamylcysteicylvaline and penicillaminic acid.

Electrophoresis. Band P was resolved into two components (P_1, P_2) with migrations identical with those of GSO_3H and cysteic acid respectively. Band Q was also resolved into two components (Q_1, Q_2) with migrations identical with those of γ-glutamylcysteicylvaline and penicillaminic acid respectively. Both components were radioactive.

Identification of components

P_1 was inferred to be GSO_3H both from its chromatographic and electrophoretic behaviour and by the release of glutamic acid, cysteic acid and glycine on hydrolysis. P_2 had properties identical with those of cysteic acid.

Component Q_2 was identified as penicillaminic acid (ββ-dimethylcysteic acid) by its chromatographic behaviour described above. The presence of radioactivity was shown to be due to the fact that it was derived from penicillin by the isolation and oxidation procedure and hence labelled after incubation with [^{14}C]valine (Halliday & Arnstein, 1956); treatment of ^{14}C-labelled penicillin in the same way as described for the isolation of substances containing amino acids and peptides produced a radioactive compound identical in properties with Q_2.

A sample of Q_1 ran as one radioactive spot, with properties very similar to γ-glutamylcysteicylvaline (I), on two-dimensional chromatography in the solvent system *C*–*A*, namely: solvent *C* (R_F 0·44; marker R_F 0·41), solvent *A* (R_F 0·07; marker R_F 0·065). Elution and hydrolysis of this material gave cysteic acid, glycine, glutamic acid, a spot with the same R_F as alanine and [^{14}C]valine (two-dimensional chromatography in solvent *A*). A further sample of Q_1, after chromatography in solvent *C* followed by elution, hydrolysis and examination of the hydrolysate by two-dimensional chromatography in solvent system *C*–*A*, gave a hydrolysis pattern identical with that described above. This result suggested that Q_1 was possibly either a pentapeptide or a smaller peptide contaminated with either GSO_3H or adsorbed amino acids. The first possibility seemed unlikely in view of the virtually identical chromatographic properties of Q_1 and (I). On electrophoresis at pH 3·5 (to remove any adsorbed neutral amino acids before hydrolysis) Q_1 behaved as one spot, with a migration identical with that of (I). Hydrolysis of a portion of Q_1, which had been further purified by electrophoresis at pH 5·0, gave mainly valine, cysteic acid and α-aminoadipic acid, together with a small amount of glutamic acid and a trace of glycine, all identified by two-dimensional electrophoresis (pH 5)–chromatography in solvent *A*. Alanine had the same R_F as α-aminoadipic acid in solvents *A* and *C* but did not migrate on electrophoresis at pH 5·0.

Quantitative examination of the hydrolysate (see Experimental section) showed that Q_1 is a tripeptide containing α-aminoadipic acid, cysteic acid and valine (Table 1, Expt. 1). The amount of glycine obtained in the first experiment is very small relative to valine, whereas in control experiments with blank paper glutamic acid was found to be eluted even from washed paper; acid hydrolysis

Table 1. *Result of quantitative hydrolysis of tripeptide isolated from mycelium of* Penicillium chrysogenum

Amino acids were estimated on paper by the method of Mandelstam & Rogers (1959). Expt. 1 refers to peptide containing [^{14}C]valine. Expt. 2 (*a*): peptide contaminated with GSO_3H. Expt. 2 (*b*): result of further purification before hydrolysis.

Expt. no.	Amino acids	Yield (μmole)	Molar ratio	Estimated total yield of peptide (μg./g. of fresh mycelium)
1	Valine	0·062	1·0	
	α-Aminoadipic acid	0·058	0·93	
	Cysteic acid	0·060	0·97	5·4
	Glutamic acid	0·014	0·23	
	Glycine	0·007	0·11	
2*a*	Valine	0·053	1·0	
	α-Aminoadipic acid	0·050	0·95	
	Cysteic acid	0·085	1·60	3·0
	Glutamic acid	0·039	0·74	
	Glycine	0·035	0·66	
2*b*	Valine	0·018	1·0	
	α-Aminoadipic acid	0·025	1·39	
	Cysteic acid	0·029	1·61	—
	Glutamic acid	0·002	0·11	
	Glycine	0·004	0·22	

of such eluates indicated the presence of glutamic acid also in bound form, which appears to be adsorbed on to other compounds during chromatography. Repeated running of Q_1 on paper during the isolation procedure could well result in contamination by the amount of glutamic acid found in the first experiment. Glutamic acid was confirmed as an artifact when the structure of the peptide was determined (Arnstein & Morris, 1960*c*). In a second experiment with peptide isolated from mycelium which had not been incubated with radioactive valine, Q_1 appeared to be contaminated with adsorbed GSO_3H in the ratio of 1·0:0·7 (see Expt. 2*a*, Table 1), although the bulk of the GSO_3H had been well separated. Further purification of the peptide decreased this contamination to less than 10%, as shown by the relative proportions of glutamic acid and α-aminoadipic acid (Table 1, Expt. 2*b*). In this estimation the proportion of valine relative to cysteic acid and α-aminoadipic acid is low, probably because the accuracy of the method was reduced by the small amounts available for this analysis compared with the others.

The quantitative estimation of α-aminoadipic acid after acid hydrolysis of the peptide may be used to calculate the amount of peptide which had been extracted from the mycelium. This calculation indicates that the peptide is present in amounts of at least 3–5 μg./g. wet wt. of mycelium (Table 1).

Some evidence was also obtained for the presence of a similar or identical peptide in extracts of a commercial strain of *P. chrysogenum*, grown in the presence of a side-chain precursor of penicillin, but the quantity was too small for full investigation.

DISCUSSION

Several investigations of sulphur compounds produced by *Penicillium chrysogenum* have been reported. Tardrew & Johnson (1958) compared the distribution of sulphate and organic sulphur in the mycelium and of penicillin and total sulphur excreted into the medium, and showed that the addition of a precursor of the penicillin side chain resulted in an increased excretion of penicillin but in a decrease in the organic-sulphur content of the mycelium. The latter finding suggests that penicillin formation depletes intracellular stores of sulphur compounds and our failure to isolate appreciable amounts of peptide from a high-penicillin-producing commercial strain may thus have been due to the presence of side-chain precursor.

Among the sulphur compounds isolated from mycelium, methionine has been obtained in relatively large amounts (approx. 1 mg./g. of mycelium) (Gordon, Numerof & Pan, 1954) and another major constituent is choline sulphate (De Flines, 1955), which appears to be present in similar amounts (2 mg./g. dry wt., Stevens & Vohra, 1955). Choline sulphate has been shown not to be an intermediate in penicillin biosynthesis (Stevens & Vohra, 1955). Both these compounds would not have been isolated by the method used in the present work, since choline sulphate is not retained by cation-exchange resins (De Flines, 1955), whereas methionine would have been adsorbed even after oxidation to the sulphone.

A minor constituent of the intracellular-sulphur fraction of *P. chrysogenum* is known to be penicillin

itself (Demain, 1957), which accounts for the presence of penicillaminic acid ($\beta\beta$-dimethylcysteic acid) in the oxidized sulphur amino acid and peptide fraction. On the assumption that fermentations contain about 10% of fresh mycelium, it may be calculated from the results of Demain (1957) that mycelium contains about 0·3 μmole of penicillin/100 g. fresh wt., compared with a total organic-sulphur content of 200 μmoles and 370 μmoles/100 g. fresh wt. in the presence and absence of side-chain precursor respectively (Tardrew & Johnson, 1958). The amount of α-aminoadipic acid present as the peptide described in this paper corresponds to approx. 0·9–1·6 μmoles/100 g. fresh wt. of mycelium. This tripeptide should therefore be considered another minor constitutent of the intramycelial-sulphur fraction. The foregoing amounts are, however, comparable with those of penicillin and hence not incompatible with a function of the peptide as an intermediate in penicillin biosynthesis. By inspection of the paper chromatograms it was clear that glutathione was present in much larger (possibly 10–50-fold) amounts than the peptide, although no quantitative estimation was made. No evidence could be obtained, however, for the presence in the mycelium of cystinylvaline or any other peptide containing both cystine and valine, and a function of the aminoadipic acid peptide in penicillin biosynthesis is therefore not unlikely.

SUMMARY

1. *Pencillium chrysogenum* WIS 51–20 F3 was incubated briefly with [^{14}C]valine and extracts of the mycelium were examined for the presence of peptides containing cystine and valine, as well as for related sulphur compounds.

2. The isolation of a tripeptide which contains α-aminoadipic acid, cysteic acid [formed from cyst(e)ine during the isolation procedure, which involved oxidation with performic acid] and valine is described.

3. After oxidation with performic acid mycelial extracts also contained cysteic acid, penicillaminic acid (derived from penicillin) and oxidized glutathione (GSO_3H).

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The Structure of a Peptide, Containing α-Aminoadipic Acid, Cystine and Valine, Present in the Mycelium of *Penicillium chrysogenum*

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In an investigation of possible intracellular intermediates in penicillin biosynthesis, a peptide containing cystine, valine and α-aminoadipic acid was isolated from the mycelium of *Penicillium chrysogenum* (Arnstein, Artman, Morris & Toms, 1960). This paper deals with a study of the amino acid sequence of this peptide and with the occurrence of free α-aminoadipic acid in *P. chrysogenum*. The possible implications of these results for penicillin biosynthesis will also be discussed. A preliminary note of these observations has been published (Arnstein, Morris & Toms, 1959).

EXPERIMENTAL

Methods

Paper electrophoresis. Electrophoretic investigations were carried out on Whatman 3MM paper with a high-

voltage apparatus capable of supplying up to 10 kv at 100 ma. The paper was cooled by close contact with a water-cooled insulated support.

Radioactivity measurements. Rough estimates of radioactivity were made for qualitative or semi-quantitative purposes either by direct counting on paper with an automatic two-dimensional scanner, or by counting of chromatogram eluates, after evaporation on to 2 cm.^{2} polythene disks, with a thin end-window Geiger–Müller tube.

Preparation of 2:4-dinitrophenyl derivatives. 2:4-Dinitrophenyl (DNP) derivatives were prepared by the method of Sanger & Thompson (1953). Dinitrophenol was removed at 60° and 0·1 mm. Hg pressure in a vacuum-sublimation apparatus.

Estimation of dinitrophenyl derivatives. The relative amounts of DNP partial-degradation products were estimated by measurement of their absorption at 360 mμ with a Hilger u.v.-absorption spectrophotometer.

Buffer solutions. Buffer concentrations are stated with respect to the anionic component. Phosphate buffers were made from sodium salts.

Materials

Cysteicylvaline, γ-glutamylcysteicylvaline and α-glutamylcysteicylvaline were obtained by performic acid oxidation of the corresponding cystine peptides (Arnstein & Morris, 1960*a*; Morris, 1960). α-Aminoadipic acid and DNP-α-aminoadipic acid were kindly given by Dr E. P. Abraham, University of Oxford.

Determination of the N-terminal amino acid

A sample of the radioactive peptide (Arnstein *et al.* 1960) (370 counts/min., equivalent to approx. 9 μg. of valine residue) was converted into the DNP derivative and hydrolysed with 6 N-HCl (1 ml.) at 110° for 18 hr. The acid solution was extracted three times with ether (3 ml. portions); all the yellow colour passed into the ether layer. The ethereal solution was evaporated to dryness *in vacuo* and the residue (in 50 μl. of ethanol) was applied to buffered paper (pH 6) and examined by two-dimensional chromatography in *tert.*-amyl alcohol–phosphate buffer (pH 6) (Blackburn & Lowther, 1951; Levy, 1954). The paper was run for 5 days in the first dimension against markers of DNP derivatives of aspartic acid, glutamic acid, glycine, valine and cysteic acid; the second dimension was run for 5 hr. against markers of DNP-glutamic acid and DNP-aspartic acid respectively.

A mixture of DNP-aspartic acid, DNP-glutamic acid and DNP-α-aminoadipic acid was subjected to electrophoresis at 86 v/cm. in 0·08 M-pyridine–acetic acid buffer, pH 5, for 1 hr., in a preliminary experiment. The unknown DNP-amino acid was eluted from the two-dimensional chromatogram, dried *in vacuo*, and the residue was subjected to high-voltage electrophoresis under the conditions just described; the three DNP-dicarboxylic amino acids were used as markers.

The unknown DNP-amino acid was eluted once again and an approximately equal amount of authentic DNP-α-aminoadipic acid was added. The mixture was then subjected to high-voltage electrophoresis at 80 v/cm. in 0·175 M-pyridine–acetic acid buffer, pH 5, for 1 hr. against DNP-α-aminoadipic acid and DNP-glutamic acid, present both singly and as a mixture.

Partial-acid-hydrolysis experiments

γ-Glutamylcysteicylvaline (1·5 mg.) was converted into the DNP-derivative in the usual way. AnalaR 12 N-HCl (0·3 ml.) was added and the mixture was placed in a stoppered siliconed test tube at 37°. Samples (30 μl.) were removed after 29, 46 and 70 hr., and, after drying *in vacuo* over P_2O_5–KOH, were examined by paper electrophoresis at 20 v/cm. in 0·08 M-pyridine–acetic acid, pH 5, for 1·5 hr. The degradation products were examined for structure by comparison with markers, both before and after development with ninhydrin, or by further degradation. The hydrolysis time for maximum concentration of partial-hydrolysis products was determined by comparison of the absorption of these products in solution at 360 mμ.

A sample of the unknown tripeptide (approx. 80 μg., as estimated by radioactivity) was converted into the DNP derivative as before. The product was subjected to electrophoresis at 80 v/cm. in 0·08 M-buffer, pH 5, for 1 hr. to check homogeneity and was then eluted from the paper with water (0·65 ml.). A portion (0·1 ml.) was evaporated on to a 2 cm.^{2} disk and counted at infinite thinness. The DNP-peptide solution was evaporated to dryness and then treated with 12 N-HCl (0·5 ml.) at 37° for 48 hr. The hydrolysate was evaporated to dryness twice with water to remove excess of HCl and the residue was subjected to electrophoresis at 46 v/cm. in 0·08 M-buffer (pH 5), for 2 hr. Valine, cysteicylvaline, cysteic acid, DNP-α-aminoadipic acid and DNP-γ-glutamylcysteicylvaline were used as markers. The degradation products were examined for structure as before. In addition, the relative concentrations of DNP products were estimated after elution from the paper by their absorption at 360 mμ, and radioactive spots were detected by scanning and then counted after elution and evaporation on to 2 cm.^{2} disks.

Isolation of α-aminoadipic acid from extracts of the mycelium of Penicillium chrysogenum. After elution of cysteic acid peptides and other acidic substances from the second column of Dowex-50 cation-exchange resin used for the isolation of sulphur-containing peptides and amino acids (Arnstein *et al.* 1960), the eluent was changed from water to 1·5 N-HCl. Fractions (20 ml.) were collected and analysed by evaporating portions (0·3 ml.) to dryness *in vacuo* and examining the residue by paper chromatography (phenol–water, 5:2, v/v, with NH_3 in the tank) and paper electrophoresis (0·08 M-pyridine–acetic acid, pH 5, 2 hr. at 25 v/cm.). Fractions nos. 53–63, which contained α-aminoadipic acid together with traces of glutamic acid and a neutral amino acid (probably glycine), were combined and freeze-dried. This material was further fractionated on a column of De-Acidite FF resin (25 g.; The Permutit Co. Ltd., London) in the acetate form. 0·5 M-Acetic acid was used as eluent and 5 ml. fractions were collected. Only two fractions (nos. 6 and 7) contained pure α-aminoadipic acid, but chromatography of the adjacent fractions (5 and 8) again on the same column yielded more pure material. α-Aminoadipic acid was identified by comparison by paper electrophoresis and chromatography as described above. In addition, butanol–acetic acid–water (63:10:27, by vol.) was used as a second solvent for paper chromatography. The fractions containing pure α-aminoadipic acid were combined and freeze-dried. The solid was dissolved in 5 N-HCl (1 ml.); the rotation of this solution was determined and the amount of α-aminoadipic acid was estimated

by quantitative paper chromatography (Mandelstam & Rogers, 1959), butanol–acetic acid–water (63:10:27, by vol.) being used as the chromatographic solvent ($[\alpha]_D$ $+14{\cdot}3 \pm 5{\cdot}7°$; *c*, 0·175). The observed rotation corresponds to a molecular rotation of $+23{\cdot}1 \pm 9{\cdot}2°$; the literature value for L-α-aminoadipic acid (*c*, 0·5–2%) is +40·3° (Greenstein, 1954).

Examination of the mycelial protein and cell-wall fraction for the presence of α-aminoadipic acid. Mycelium of *Penicillium chrysogenum* WIS 51–20 F3 was defatted with boiling ethanol, ethanol–ether (3:1, v/v) and ether and extracted with hot 5% trichloroacetic acid. The extracted protein-rich residue (60 mg.) was hydrolysed with 6N-HCl (0·6 ml.) for 18 hr. at 105°. After addition of a little water the hydrolysate was filtered and evaporated to dryness *in vacuo* over KOH. The residue was dissolved in water (0·4 ml.) and portions (10 μl., equivalent to 1·5 mg. of extracted protein + cell-wall fraction) were examined by electrophoresis on Whatman no. 3MM paper (0·08M-pyridine–acetic acid, pH 5, 53 v/cm. for 75 min.), amino acids being detected by spraying with 0·1% ninhydrin in butanol containing 2% of water. Good separation of α-aminoadipic acid, glutamic acid and aspartic acid markers was obtained, but the hydrolysate contained only glutamic acid and aspartic acid. Since less than 1·5 μg. of α-aminoadipic acid is visible, the presence of 0·1% of α-aminoadipic acid in the extracted mycelium would have been detected by this procedure.

RESULTS

N-terminal amino acids

The unknown DNP-amino acid ran as one spot in both dimensions on two-dimensional chromatographic analysis. In the first dimension it was not clearly separated from DNP-glutamic acid or DNP-aspartic acid. In the second dimension, however, the material moved slightly slower than DNP-glutamic acid, which itself was slower than DNP-aspartic acid. This result suggested that the unknown was probably a higher homologue of these acids, for example, α-aminoadipic acid. In so far as the peptide had already been shown to consist of one residue of each of cysteic acid, valine and α-aminoadipic acid (Arnstein *et al.* 1960), the last-named was almost certainly *N*-terminal. Preliminary electrophoresis of DNP-glutamic acid, DNP-aspartic acid and DNP-α-aminoadipic acid showed that these compounds were completely separated at pH 5. Under the same conditions the unknown material migrated 17·1 cm. (identical with that of DNP-α-aminoadipic acid marker) and was clearly not DNP-aspartic acid (migration 22·5 cm.). The migration of the two DNP-glutamic acid markers, however, was not identical (16·5 and 18·2 cm.), presumably caused by uneven wetting of the paper. In the final experiment the mixture of the unknown and DNP-α-aminoadipic acid ran as one spot, whereas the mixture of DNP-α-aminoadipic acid and DNP-glutamic acid separated into two distinct components. These results demonstrated conclusively that the *N*-terminal amino acid of the tripeptide was α-aminoadipic acid.

Partial-hydrolysis experiments

Partial hydrolysis of DNP-γ-glutamylcysteicylvaline gave three yellow components, corresponding, in order of increasing electrophoretic mobility, to DNP-glutamic acid, unchanged DNP-peptide and an unknown DNP product. The latter was shown, by elution, hydrolysis and subsequent paper chromatography in butanol–acetic acid–water (63:10:27), to be DNP-γ-glutamylcysteic acid. After development with ninhydrin three blue spots were seen in addition to the three yellow components described above. The ninhydrin-positive spots were shown, by comparison with markers, to be valine, cysteicylvaline and cysteic acid respectively. The order of increasing migration of the six degradation products was valine, cysteicylvaline, DNP-γ-glutamylcysteicylvaline and DNP-γ-glutamylcysteic acid.

The $\epsilon_{max.}$ values of DNP-γ-glutamylcysteic acid at 29, 46 and 70 hr. were 0·06, 0·070 and 0·058 respectively, indicating that a hydrolysis time of about 46 hr. was optimum for maximum production of this degradation product.

Electrophoresis of the partial hydrolysate of the oxidized natural peptide gave a very similar result to that described above. The electrophoresis pattern is shown in Fig. 1. The six degradation

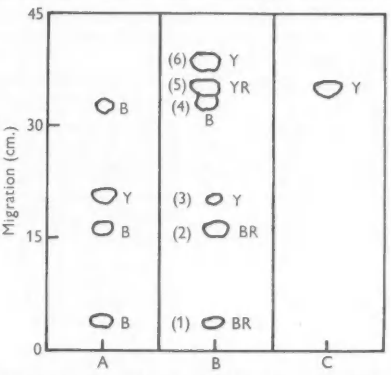


Fig. 1. Electrophoresis of DNP-peptide for 2 hr. at 3·5 kv in 0·08M-pyridine–acetic acid buffer, pH 5, after treatment with 12N-HCl for 48 hr. at 37°. Result is shown in column B. Markers: column A (in order of increasing mobility), valine, cysteicylvaline, DNP-α-aminoadipic acid and cysteic acid; column C, DNP-γ-glutamylcysteicylvaline. The paper was scanned for radioactivity and then developed with ninhydrin. For identification of the products numbered in parentheses, see text. Y, Yellow; B, blue; R, radioactive.

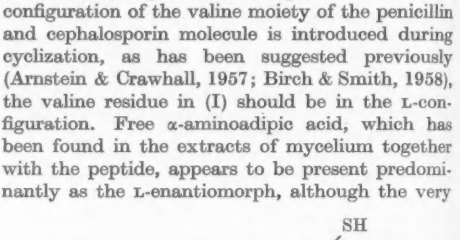
products were: (1) valine, (2) cysteicylvaline, (3) DNP-α-aminoadipic acid, (4) cysteic acid, (5) unchanged DNP-peptide, (6) DNP-α-aminoadipylcysteic acid, as shown below. Valine and cysteicylvaline were identified by their mobility compared with authentic specimens and by the presence of radioactivity (detected by scanning). DNP-α-aminoadipic acid had the same mobility as the authentic marker, and the unchanged DNP-peptide was radioactive and had a mobility identical with that of DNP-γ-glutamylcysteicylvaline. The structure of compound (6) was inferred from its mobility, which was very similar to that of DNP-γ-glutamylcysteic acid, and from the absence of radioactivity, showing that valine had been lost from the starting material. Products (5) and (6), after they had been eluted, evaporated to dryness and taken up in water (0·5 ml.), had $\epsilon_{max.}$ values at 360 mμ of 0·495 and 0·620 respectively; the radioactivity contained in these products was 103·2 and 6·3 counts/min. respectively. Since (5) is present in smaller amount than (6) and yet has more than 16 times the radioactivity, the very small activity in (6) can be neglected, thus confirming the absence of valine. These results indicate that the amino acid sequence of the tripeptide is α-aminoadipylcysteicylvaline.

Electrophoresis of free tripeptide

The free peptide had an electrophoretic mobility (3·0 cm.), identical with the mobilities of α- and γ-glutamylcysteicylvaline at pH 2·1. At pH 3·5, however, the α-glutamyl tripeptide had a migration of 4·2 cm., whereas both the γ-glutamyl and aminoadipyl tripeptides had an increased and identical migration of 10·0 cm. This behaviour shows that, analogously to the γ-glutamyl peptide, the α-carboxyl group of the α-aminoadipic acid is free and therefore the latter must be linked to the rest of the molecule by its δ-carboxyl group. Thus the structure of the tripeptide isolated from mycelial extracts after oxidation with performic acid must be δ-(α-aminoadipyl)cysteicylvaline.

DISCUSSION

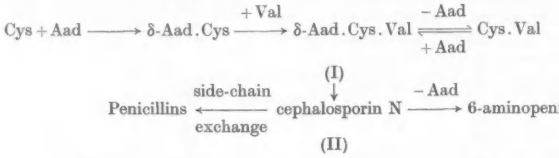
The evidence presented in this paper indicates that the structure of the α-aminoadipic acid peptide which occurs in the mycelium of *P. chrysogenum* is either (I) or its disulphide. Unfortunately, insufficient material was available for determination of the optical configuration of the constituent amino acids. The possible biogenetic relationship of (I) to cephalosporin N (II, Newton & Abraham, 1954) is, however, sufficiently indicated by the similarity in the amino acid sequence and by the linkage of the α-aminoadipic acid through its δ-carboxyl group in both compounds. If the D-configuration of the valine moiety of the penicillin and cephalosporin molecule is introduced during cyclization, as has been suggested previously (Arnstein & Crawhall, 1957; Birch & Smith, 1958), the valine residue in (I) should be in the L-configuration. Free α-aminoadipic acid, which has been found in the extracts of mycelium together with the peptide, appears to be present predominantly as the L-enantiomorph, although the very small amount available considerably decreased the accuracy of measurement of the optical rotation. Since the α-aminoadipic acid side chain of cephalosporin N has the D-configuration (Newton & Abraham, 1954), α-aminoadipic acid in (I) could be either the D- or the L-form depending on whether the optical configuration is changed before or after cyclization.



(I)

(II)

A possible pathway for penicillin biosynthesis is summarized in the following scheme (Cys, cystine or cysteine; Val, valine; Aad, aminoadipic acid):



This scheme would account for the presence of (I) in amounts comparable with the intracellular concentration of penicillin (Arnstein *et al.* 1960) and for the direct utilization of labelled cyst(e)inylvaline for penicillin biosynthesis (Arnstein & Morris, 1960*b*), presumably by incorporation of the labelled dipeptide into (I) by a transpeptidation reaction similar to that involved in the incorporation of cysteinylglycine into glutathione (Fodor, Miller, Neidle & Waelsch, 1953). By analogy with

glutathione, new synthesis of (I) would, however, take place with the intermediary formation of α-aminoadipylcystine, which would account for the observation that the conversion of labelled cystinylvaline into penicillin was quantitatively limited (Arnstein *et al.* 1960).

Cyclization of (I) would give rise to cephalosporin N, which has, however, not yet been detected in fermentations of *P. chrysogenum* (see Arnstein, 1957). Since cephalosporin N is postulated only as an intermediate, it would be expected to be present in very small amounts, possibly only intracellularly, and a careful investigation of penicillin-like compounds in the mould mycelium would therefore be of interest. The final step of penicillin biosynthesis would involve exchange of the α-aminoadipic acid side-chain of cephalosporin N for a carboxylic acid, e.g. phenylacetic acid with benzylpenicillin. Such a reaction seems not unlikely in view of the known side-chain transfer between benzyl- and phenoxymethyl-penicillin (Peterson & Wideburg, 1958). It is also suggested that the mould *Cephalosporium*, which synthesizes cephalosporin N, but is unable to produce benzylpenicillin even in the presence of phenylacetic acid (see Arnstein, 1957), may lack the appropriate transferase. On the other hand, 6-aminopenicillanic acid, which is biosynthesized by *Penicillium chrysogenum* in the absence of phenylacetic acid or other side-chain precursors (Batchelor, Doyle, Nayler & Rolinson, 1959), may arise by loss of the side chain of cephalosporin N and would therefore be a shunt product rather than an intermediate in penicillin biosynthesis.

Although there are still many gaps in the evidence for this scheme of penicillin biosynthesis, experimental verification of most of its implications would appear to be feasible without undue difficulty.

SUMMARY

1. The *N*-terminal group of a peptide which occurs in the mycelium of *Penicillium chrysogenum* has been shown to be α-aminoadipic acid and the amino acid sequence has been determined by partial hydrolysis with acid.

2. The structure of the peptide is δ-(α-aminoadipyl)cyst(e)inylvaline.

3. α-Aminoadipic acid has also been isolated from mycelium and appears to be present mainly as the L-enantiomorph.

4. The possible function of α-aminoadipic acid, of the peptide and of cephalosporin N in penicillin biosynthesis is discussed.

We are grateful to Dr E. P. Abraham, F.R.S., for samples of α-aminoadipic acid and its dinitrophenyl derivative and we should like to thank Mr E. J. Toms for skilled technical assistance.

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Biochem. J. (1960) **76**, 362

Action of Streptomycin on *Vibrio cholerae*

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Arora & Krishna Murti (1955) showed that broad-spectrum antibiotics inhibit oxygen uptake by *Vibrio cholerae* cells in the presence of the tricarboxylic acid-cycle intermediates, but have no perceptible effect on the corresponding oxidations in animal-tissue homogenates. The availability of a *V. cholerae* strain sensitive to streptomycin and a resistant mutant derived from it has made it possible to extend this work, which was initiated originally with the object of elucidating the mechanism of action of antibiotics (Krishna Murti, 1959), to a more detailed evaluation of streptomycin inhibition. The results presented in this paper support our earlier findings and indicate that succinic dehydrogenase and fumarase are among the enzymes inhibited by streptomycin.

METHODS AND MATERIALS

Organisms. A strain of *V. cholerae* Inaba S^S (InSS), and a streptomycin-resistant mutant derived from it, Inaba S^R (InSR), and Ogawa 82/L were kindly supplied by Dr K. Bhaskaran of this Institute.

Medium. Nutrient agar made of Lab-Lemco (1%, w/v), Difco Peptone (1%, w/v), Marmite (0·5%), NaCl (0·5%) and Difco agar (2·5%) was used throughout for the maintenance and growth of the organisms. Before the addition of agar and before autoclaving, the broth was adjusted to pH 8·2 (glass electrode) with 2N-NaOH. Subculturing on this medium was carried out every fortnight.

Washed suspensions. Roux flasks containing 80 ml. of nutrient agar were seeded with an overnight growth of the organism in 10 ml. of nutrient broth. The cells were harvested with ice-cold 0·85% NaCl solution after 18 hr. growth at 37°. Since previous studies of Iyer, Dudani, Krishna Murti & Shrivastava (1953–54) had emphasized the importance of aqueous NaCl for washing and preparing cells of vibrios for enzymic studies, the organisms were washed three times with cold 0·85% NaCl solution on the centrifuge (1500 *g* for 20 min. at 5°) and finally suspended in 0·85% NaCl solution. For manometric studies the opacity of the suspension was adjusted to a Klett reading of 250 (red filter 660 mμ). Dry-weight determination of random samples of the suspensions showed that at this opacity value they contained 1 mg. bacterial dry wt./ml. Cell suspensions thus prepared showed a negligible endogenous O_2 uptake.

Cell-free extracts. These were prepared by sonic oscillation (at 25 kcyc./sec. with an output amperage of 2·5A) from freshly harvested cells suspended in cold 0·85% NaCl solution in a 500w Mullard Magneto strictor oscillator. The cell suspension was kept in a stainless-steel inducer flask which was cooled by continuous circulation of chilled water in an outer vessel. Cells subjected to impulses below 15 kcyc./sec. showed little lysis. A 15 min. exposure at 25 kcyc./sec., however, lysed the cells completely, as was evident by the clearing of turbidity and subsequent staining, which revealed less than 1% of Gram-negative bodies. The resulting material was centrifuged for 15 min. at 1500 *g* at 5° to remove unbroken cells and debris and the cell-free supernatant was stored in the frozen state at −10°.

An extract prepared as described was, however, devoid of succinic-dehydrogenase and fumarase activities. To obtain a cell-free preparation rich in these two enzymes, the following procedure was evolved. Cultures grown for 22 hr. on medium in 10 Roux flasks were harvested and washed as described before. The sedimented cells were maintained at −10° for 12 hr. The thawed mass was ground with acid-washed carborundum (200 mesh, Delhi Chemical Works, Delhi) in a chilled mortar and extracted with two 5 ml. portions of cold 0·85% NaCl solution. The extracts were freed from unbroken cells, cell debris and abrasive by centrifuging at 2000 *g* for 20 min. and precipitated at 5° by saturation with $(NH_4)_2SO_4$. The precipitated material was centrifuged off (800 *g* for 15 min. at 0°) dispersed in 5 ml. of cold 0·85% NaCl solution and dialysed against running cold distilled water for 18 hr. The volume of the suspension after dialysis was made up to 10 ml. with ice-cold glass-distilled water and clarified by centrifuging (800 *g* at 0° for 15 min.). The residue possessed appreciable succinic-dehydrogenase and fumarase activities and was used as such in inhibition studies.

Buffers. These were 0·2M-Na_2HPO_4–KH_2PO_4, pH 8·0, and 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 8·6.

Manometry. Air-filled Warburg manometers were used at 37°. Single-side-arm flasks contained buffer, cell suspension and drug in the main compartment and the substrate in the side arm. The total volume was maintained at 3 ml. The centre well always contained 0·2 ml. of 10% KOH with a 2 cm.² filter-paper roll.

Enzyme-assay methods. Nitrate reduction and acetoin production were estimated according to Clarke & Cown (1952), succinic dehydrogenase by methylene-blue reduction (Bonner, 1955) or tetrazolium reduction (Srikantan & Krishna Murti, 1955), fumarase by the optical method devised by Racker (1950), β-galactosidase according to Lederberg (1950) as modified by Kuby & Lardy (1953) and nucleases according to Sagar, Krishna Murti & Shrivastava (1958) after measurements of extinction at 260 mμ of the reaction products.

Glyoxalate production was tested in evacuated Thunberg tubes. Cell-free extract, buffer and inhibitor were placed in the main tube and *iso*citrate in the hollow bent stopper. In a typical assay the constituents were 100 μmoles of tris, 40 μmoles of DL-*iso*-citrate and 0·5 ml. of cell-free extract in

a total volume of 2 ml. The tubes were evacuated, closed and the contents mixed. After a 2 hr. incubation period at 37° the glyoxalate formed was fixed as a dinitrophenylhydrazone and identified by paper chromatography (Saxena, Krishna Murti & Shrivastava, 1956) with butan-1-ol saturated with 0·4 N-NH_3 solution as the solvent for development.

Analytical methods. Protein concentration in cells and cell-free extracts was measured after precipitation with a 5% trichloroacetic acid solution according to Lowry, Rosebrough, Farr & Randall (1951). Nucleic acid concentration was estimated by measuring extinction at 260 mμ. Dihydrostreptomycin was estimated by the nitroprusside colour according to Monastero (1952).

Incorporation experiments. The medium for incorporation of labelled glycine contained Casamino acids (Difco) supplemented with L-tryptophan and L-cystine, purines, pyrimidines, buffered salt solutions (Gale, 1947), cells and labelled glycine diluted with carrier glycine (5 mg./ml.). This was made up by mixing 16 ml. of salts–buffer, pH 8·0, 7 ml. of amino acid solutions containing 40 mg. of Casamino acids and 20 mg. each of L-tryptophan and L-cystine/ml. (pH 7·5), 2·8 ml. of purine–pyrimidine solution (1 mg. each of adenine sulphate, hypoxanthine, xanthine, guanine–HCl, thymine, and uracil/ml.), 7 ml. of 10% glucose and 10 ml. of labelled glycine diluted with carrier. This medium (6 ml.) thus constituted is referred to as 'incorporation medium' and was mixed with 3 ml. of cell suspension and 1 ml. of water or drug as indicated. The concentration of cells in the medium was adjusted so as to give 10 mg. of cell mass/ml. This concentration of cell mass was found to be the minimum required for the subsequent isolation and plating of labelled protein. The mixture kept in 500 ml. Erlenmeyer flasks was shaken in a rotary shaker at 37°. At intervals 2 ml. portions were added to 1 ml. of cold 20% trichloroacetic acid and centrifuged at 1500 *g* for 10 min. The sedimented cells were extracted with 10 ml. of 5% trichloroacetic acid containing 10 mg. of unlabelled glycine/ml., by placing the tubes in boiling water for 10 min., and the protein was sedimented by centrifuging at 1500 *g* for 15 min. The trichloroacetic acid extraction was repeated, the protein pellet finally dispersed in 0·5 ml. of aqueous NH_3 (sp.gr. 0·92) and plated on planchets; a drop of an aqueous solution (1% w/v), of dodecylquaternarypyridinium (Imperial Chemical Industries Ltd.) was used to facilitate the plating. The protein films were dried under an infrared lamp and counted in a thin-window Geiger–Müller counter. Counts up to 10 000 were made in active samples and the total count for a 30 min. period was recorded for weaker samples. Since 'infinitely thin' films of radioactive material were used for counting, no correction for self-absorption was made.

Carrier-free [2-^{14}C]glycine solution (1 μc/ml.), made by dissolving in 100 ml. of glass-distilled water, 1·94 mg. of the radioactive powder (The Radiochemical Centre, Amersham, Bucks.) having a total activity of 0·1 mc, was kindly supplied by Dr S. C. Agarwala of this Institute.

Protein synthesis by cells. The medium was essentially similar to the one used in incorporation experiments but the proportion of the individual constituents was different. It was made of 5 ml. of salts–buffer, pH 8·0, 0·8 ml. of Casamino acids supplemented with L-tryptophan and L-cystine, 0·2 ml. of purine–pyrimidine mixture, 1·0 ml. of 1% glucose solution and 1·5 ml. of water or drug solution. To this mixture was added 5 ml. of cell suspension to give a final concentration of 1 mg. of cell mass/ml. Flasks containing cell suspensions were shaken in a rotary shaker at 37°. Samples (1 ml.) were withdrawn at the specified intervals and pipetted into 1 ml. of 10% trichloroacetic acid. The tubes containing the precipitated cells were kept in boiling water for 10 min. and centrifuged for 15 min. at 2500 *g*. The supernatants were discarded, the residue was dissolved in alkaline copper reagent and their protein content estimated. Bovine plasma albumin (Armour Laboratories, Chicago, Ill., U.S.A.) was used as standard.

Isolation of nucleic acids. Freshly harvested and washed cells were suspended in 30 ml. of arsenate–citrate–0·85% NaCl medium (0·05 M-sodium dihydrogen arsenate and 0·1 M-sodium citrate in 0·85% NaCl solution, pH 7·0) and mixed with 5 ml. of 10% sodium deoxycholate solution. The suspension became very viscous and the pH rose to 7·5. The gel was washed into a 500 ml. Pyrex bottle with 30 ml. of arsenate–citrate–NaCl and mixed with 20 ml. of $CHCl_3$ and 6 ml. of amyl alcohol. The bottle was shaken mechanically for 30 min. at 5° and the resulting suspension was centrifuged at 2500 *g* for 10 min. The process of deproteinization was repeated three times to yield an aqueous solution of nucleic acid fairly free from protein, the nucleic acids were precipitated with 20% cetylpyridinium bromide solution (Jones, 1953). The stringy nucleic acid complex could be taken up on a glass rod. The complex was dispersed in NaCl solution and shaken repeatedly with $CHCl_3$ to remove the cetylpyridinium bromide. Absorption spectra of NaCl solutions of this preparation gave curves similar to that of yeast ribonucleic acid and sperm deoxyribonucleic acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) with a characteristic absorption peak at 260 mμ and no hump at 280 mμ. The solution was used as such for preparing the dihydrostreptomycin complexes by adding dihydrostreptomycin solution (100 mg./ml.) dropwise. The precipitated material was washed with 0·85% NaCl solution to remove the drug. The precipitate obtained from 2 ml. of the original solution was homogenized in a Potter–Elvehjem homogenizer with 5 ml. of 0·2 M-sodium acetate, pH 7·5, and used as substrate for the action of crystalline ribonuclease and deoxyribonuclease (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Drug sensitivity of the organism. Assay of drug sensitivity or resistance was carried by visually recording growth of InSS and InSR in nutrient broths (10 ml.) containing dihydrostreptomycin (0–2 mg./ml.). Growth of InSS was completely suppressed by concentration of dihydrostreptomycin as low as 2 μg./ml., whereas InSR grew very well in concentrations up to 2 mg./ml.

RESULTS

Effect of streptomycin on acetate oxidation. Volumes of oxygen used (μl./mg. dry wt./hr.; Q_{O_2} values) for acetate oxidation by fresh washed suspensions of InSS and InSR are given in Table 1. A perceptible inhibitory effect is produced even by the lowest concentration of dihydrostreptomycin used; the resistant strain is not inhibited by five times the concentration of the drug that causes 30% inhibition in InSS.

Effect of streptomycin on oxidation of Krebs-cycle intermediates. The Q_{O_2} values are given in Table 2. Dihydrostreptomycin considerably retarded the rate of oxidation of all the substrates by InSS excepting malate and lactate. Citrate was not included, because resting cells of *V. cholerae* are not permeable to citrate (Krishna Murti & Shrivastava, 1955*a*). The effect of various concentrations of dihydrostreptomycin and malate on the Q_{O_2} values are given in Tables 3 and 4. The consistent failure to observe inhibition with malate suggests that dihydrostreptomycin inhibits fumarase. Insensitivity of lactate oxidation to dihydrostreptomycin may be due to the conversion of lactate into malate by lactic dehydrogenase and 'malic enzyme'.

Effect of streptomycin on the oxidation of amino acids. The Q_{O_2} values are given in Table 5. The drug exerts an appreciable depressing action on the oxidation of the amino acids tested only by InSS.

Age of harvested cells and the degree of inhibition by streptomycin. Oxidation of acetate, succinate and fumarate was followed with freshly prepared cell suspensions of InSS and periodically thereafter with the same suspension stored at 8°. The Q_{O_2} values are given in Table 6. Attention is particularly drawn to the gradual diminution in the degree of inhibition of acetate oxidation by dihydrostreptomycin with aging of cells and the persistence of inhibition of fumarate and succinate oxidation even after 96 hr. of storage.

Effect of streptomycin on the rates of oxidation of various substrates. The rates of oxidation of a number of substrates by InSS and InSR grown under different conditions (cf. legends to Figs. 1–4) were compared in order to find out whether resistance leads to alterations in metabolic activity. Results of some typical experiments are presented graphically in Figs. 1–4. Dihydrostreptomycin always inhibited oxidation of acetate, succinate and fumarate by InSS. This inhibition is evident from the time the substrate is added to the reaction system. Oxygen uptake in the presence of glucose, mannose and maltose is, however, inhibited by the drug only after a lag period of 30–60 min. InSR grown with or without dihydrostreptomycin in the medium showed almost the same activity on all the substrates tested as strain InSS. Results presented in Fig. 4 show a lag period of about 30 min. before oxidation of histidine commences. Presumably the cells are adapted to use this substrate. Although dihydrostreptomycin inhibits oxygen uptake by InSS on this amino acid, the results do not conclusively indicate inhibition of adaptation.

Effect of dihydrostreptomycin on cell-free extracts

Production of acetoin. Streptomycin dependence and resistance in *Salmonella typhosa* are accompanied by the cell's gaining the ability to produce acetylmethylcarbinol which the parent sensitive strains were unable to do (Reitmann & Iverson, 1953–54). We therefore examined whether a similar change occurs in *V. cholerae*; acetylmethylcarbinol production was followed in a number of assay systems with both cell suspensions and extracts of InSS and InSR and no positive test was observed.

Nitrate reduction. Reduction of nitrate by cell-free extracts was followed for a period of 30 min. and no significant difference could be observed between the activities of InSS and InSR.

Production of glyoxalate. Suspensions were incubated in evacuated Thunberg tubes, and a dinitrophenylhydrazone was isolated which was identified by paper chromatography as that of glyoxalate. No difference could be established in the rate of production of this compound by extracts of InSS and InSR. This would suggest the presence of the glyoxalate shunt in both the strains. Since malate is formed in certain micro-organisms from acetate via glyoxalate (Kornberg & Madsen, 1958) the glyoxalate shunt may be involved in resistance.

Action of dihydrostreptomycin on fumarase and succinic dehydrogenase. For following the action of dihydrostreptomycin on succinic dehydrogenase

Table 1. *Oxidation of acetate by InSS and InSR*

The flasks contained 100 μmoles of phosphate, pH 8·0, 5 μmoles of sodium acetate, cells equivalent to 1 mg. cell mass and dihydrostreptomycin as indicated, in a total volume of 3 ml. The substrate was tipped in from the side arm after pre-incubating the cells with drug for 30 min.

	Concn. of dihydrostreptomycin (μg./ml.)					
	0	16·7	33·3	50·0	66·7	83·3
	Q_{O_2}					
InSS	62	44	31	39	34	34
InSR	77	78	78	79	77	78

Table 2. *Oxidation of tricarboxylic acid-cycle intermediates and lactate by InSS and InSR*

The conditions are as in Table 1 with 5 μmoles of the substrates as shown; dihydrostreptomycin (16·7 μg./ml.) present (+); absent (−). The values are given as Q_{O_2} values.

	*iso*Citrate		α-Oxo-glutarate		Succinate		Fumarate		Malate		Lactate	
	−	+	−	+	−	+	−	+	−	+	−	+
InSS	80	62	20	8	72	12	100	12	70	71	114	113
InSR	80	82	24	24	78	80	98	102	68	69	115	113

0·5 ml. of enzyme extract was mixed with 0·5 ml. of 0·2 M-potassium phosphate buffer, pH 8, and incubated at room temperature with 0·2 ml. of water (control) or 0·2 ml. of dihydrostreptomycin (1 mg./ml.) for 30 min.; 0·5 ml. of triphenyl tetrazolium chloride (0·05%, pH 7·5) and 0·2 ml. of 0·5 M-sodium succinate were added and incubated for a further period of 4 hr. at 37°. The formazan colour formed was extracted with a total volume of 6 ml. of toluene–acetic acid (1:1). A portion (1·5 ml.) of the coloured layer was diluted with 1·5 ml. of the same solvent mixture and the extinction was read in cuvettes (optical path 1 cm.) at 495 mμ in a Unicam spectrophotometer. The extinction values for InSS were 0·1 for control and 0·055 with dihydrostreptomycin. The extinction values in InSR were 0·15 and 0·145 respectively. A 45% inhibition of succinic dehydrogenase is brought about by dihydrostreptomycin on InSS without affecting the enzyme made from InSR.

To study the action of dihydrostreptomycin on fumarase, 1 ml. of enzyme extract was mixed with 1 ml. of 0·2 M-phosphate buffer, pH 8·0, and incubated with 0·2 ml. of water (control) or 0·2 ml. of dihydrostreptomycin (1 mg./ml.) at 30° for 30 min. A portion (1 ml.) of 0·1 M-sodium fumarate was added to the mixture and 1 ml. portions were pipetted into 1 ml. of 10% trichloroacetic acid immediately and 15 min. after the addition of fumarate. The proteins were centrifuged down and the extinction of clear supernatants (diluted 1:5) was read in silica cuvettes (1 cm. optical path) at 300 mμ. The decrease in extinction of the control

Table 3. *Effect of dihydrostreptomycin concentration on oxidation of malate by InSS and InSR*

The conditions are as in Table 1; the concentration was 5 μmoles of substrate/flask.

	Concn. of dihydrostreptomycin (μg./ml.)					
	0	33·3	66·6	100	133·3	166·7
	Q_{O_2}					
InSS	70	72	78	70	68	72
InSR	68	69	68	69	70	68

Table 4. *Effect of substrate concentration upon oxidation of malate by InSS and InSR*

The conditions are as in Table 1.

	Concn. of malate (μmoles/flask)				
	5	10	20	40	50
	Q_{O_2}				
InSS	80	123	128	147	170
InSR	84	122	126	148	172

Table 5. *Oxidation of amino acids by InSS and InSR*

Conditions were as given in Table 1. Substrate: 20 μmoles/flask. +, Dihydrostreptomycin present (100 μg./ml.); −, dihydrostreptomycin absent. Results are given as uptake of oxygen.

		L-Aspartate			Glycine			DL-Serine			L-Cysteine			L-Glutamate			DL-Threonine		
Strain	Time (min.)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)	−	+	Inhibition (%)
InSS	60	63	33	48	26	26	0	35	22	37	60	59	0	15	15	0	28	22	22
	90	90	40	55	38	30	22	50	32	36	84	69	18	22	17	29	41	30	27
	120	118	49	58	51	33	36	72	41	43	94	68	29	33	17	50	58	37	36
	180	172	78	56	74	31	60	100	52	48	160	68	58	42	18	70	82	42	49
InSR	60	138	130	0	5	65	0	146	151	0	42	45	0	—	—	—	—	—	—
	90	200	202	0	84	88	0	168	170	0	64	65	0	—	—	—	—	—	—
	120	228	226	0	92	96	0	182	188	0	84	88	0	—	—	—	—	—	—
	180	248	250	0	112	120	0	212	220	0	108	112	0	—	—	—	—	—	—

for InSS was 0·1, whereas the dihydrostreptomycin-treated system showed no decrease in extinction during the period, indicating complete inhibition of fumarase activity. In contrast the fall in extinction was 0·15 in both the control and dihydrostreptomycin-treated system in InSR, indicating that the drug has no action on the fumarase of InSR.

Susceptibility of dihydrostreptomycin–nucleic acid complexes to the hydrolytic action of ribonuclease and deoxyribonuclease

0·2 M-Acetate homogenates (1 ml.) of dihydrostreptomycin–nucleic acid complexes prepared as described in the Methods and Materials section was mixed with 300 μg. of deoxyribonuclease or ribonuclease for 60 min. at 37°. The uncombined nucleic acids were precipitated with 1 ml. of MacFadyan's (1934) reagent and the extinctions of the supernatants measured at 260 mμ after appropriate dilution. Extinction values of the supernatants of InSS were 0·60 and 0·27 respectively for ribonuclease and deoxyribonuclease. The corresponding values for InSR were 1·60 and 1·40. From this it would appear that nucleic acid–dihydrostreptomycin complexes derived from InSS and InSR are both susceptible to the action of the enzymes. More material absorbing at 260 mμ is solubilized by the enzymes from the dihydrostreptomycin complex of InSR than from the corresponding complex of InSS.

Table 6. *Oxidation of acids in InSS as affected by aging of cells*

Conditions are as given in Table 1. Substrate: 10 μmoles/flask, +, Dihydrostreptomycin present (16·6 μg./ml.); −, dihydrostreptomycin absent. The age of cells represents the period stored at 8° after harvesting. Results are given as Q_{O_2} values.

Age of cells (hr.)	Acetate		Fumarate		Succinate	
	−	+	−	+	−	+
Fresh	126	98	118	14	120	12
24	109	90	116	14	116	23
48	74	68	98	32	98	36
96	72	72	54	30	52	30

Action of dihydrostreptomycin on protein synthesis and incorporation of ^{14}C

V. cholerae does not ordinarily ferment lactose (Wilson & Miles, 1955) and attempts to induce formation of β-galactosidase in InSS and InSR under a variety of conditions were unsuccessful. The Ogawa 82/L strain grew very poorly on nutrient agar even after 48 hr. incubation. Notwithstanding this sparse growth, freshly harvested cells of this strain were used in exploratory induction experiments and dihydrostreptomycin was found to inhibit enzyme formation. Since the results

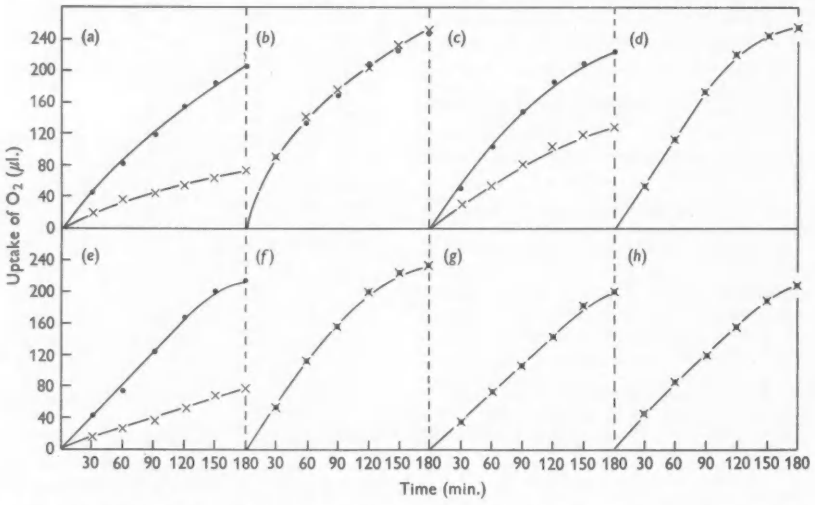

Fig. 1. Uptake of O_2 by InSS and InSR. The flasks contained: cells (1 mg. dry wt.), 20 μmoles of substrate, 33 g. of dihydrostreptomycin (present, ×; absent, ●), 100 μmoles of phosphate buffer, pH 8·0. The substrate was tipped in from the side arm after 30 min. pre-incubation. (*a*) InSS + succinate; (*b*) InSR + succinate; (*c*) InSS + acetate; (*d*) InSR + acetate; (*e*) InSS + fumarate; (*f*) InSR + fumarate; (*g*) InSS + malate; (*h*) InSR + malate.

were not conclusive, the effect of dihydrostreptomycin was studied on protein synthesis measured by increase in cellular nitrogen. The results of a typical experiment are summarized in Table 7. Protein synthesis is inhibited by dihydrostreptomycin in InSS whereas in InSR it proceeds even in the presence of the drug. It is also apparent that the net synthesis of protein/ml. of medium is greater in InSR than in InSS.

The growth rates of the two strains in nutrient broth under identical conditions were, however, comparable and the protein and nucleic acid contents of cells on dry-wt. basis at 18–24 hr. growth did not show any quantitative differences between InSS and InSR. Analysis of cell suspensions of InSS and InSR of equal opacity value had a protein content of 500–530 μg./mg. dry wt. and extinction values at 260 mμ of trichloroacetic acid extracts of cells of InSS and InSR were comparable.

Action of dihydrostreptomycin on incorporation of [^{14}C]glycine into the trichloroacetic acid-precipitable fraction of cells

Incorporation of labelled amino acid was followed for a period of 3 hr. and the results have been presented in Fig. 5. The ^{14}C-incorporation data run almost parallel to results on protein synthesis. Here again rate of incorporation of ^{14}C/ml. of suspension was greater in InSR than in InSS. A comparison of the data for protein synthesis given in Table 7 and the data for incorporation shown in Fig. 5 reveals that whereas chloramphenicol stops protein synthesis completely, incorporation of labelled glycine is inhibited only 50 %.

DISCUSSION

The results presented in this paper establish clearly that streptomycin exerts its primary cyto-

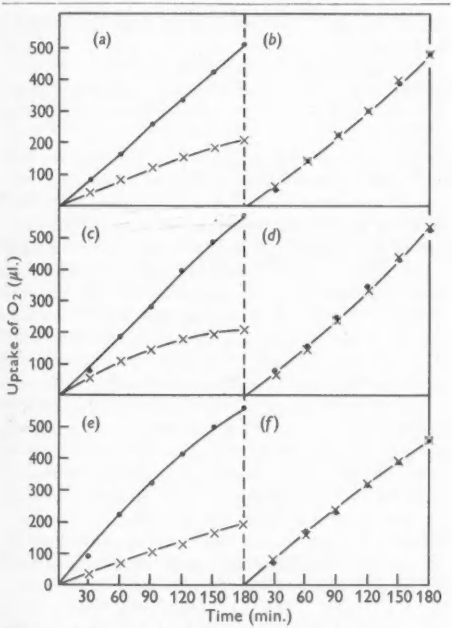

Fig. 2. Uptake of O_2 by InSS and InSR. InSR were grown on nutrient agar containing 500 μg. of dihydrostreptomycin/ml. The conditions are as for Fig. 1. (*a*) InSS + acetate; (*b*) InSR + acetate; (*c*) InSS + fumarate; (*d*) InSR + fumarate; (*e*) InSS + succinate; (*f*) InSR + succinate.

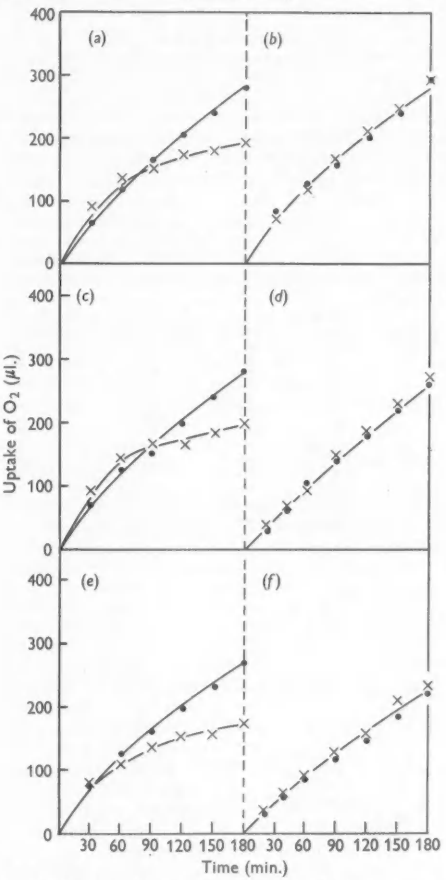

Fig. 3. Uptake of O_2 by InSS and InSR. InSR were grown on nutrient agar containing 500 μg. of dihydrostreptomycin/ml. The conditions were as for Fig. 1. (*a*) InSS + glucose; (*b*) InSR + glucose; (*c*) InSS + maltose; (*d*) InSR + maltose; (*e*) InSS + mannose; (*f*) InSR + mannose.

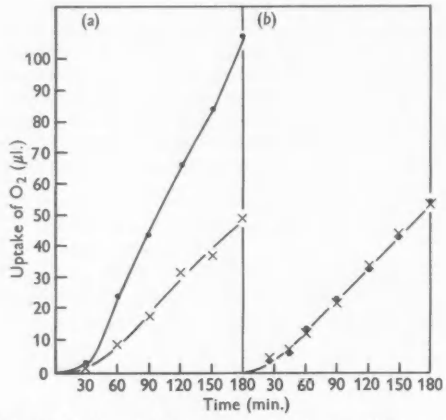

Fig. 4. Action of dihydrostreptomycin on oxidation of histidine. InSR were grown on nutrient agar containing 500 μg. of dihydrostreptomycin/ml. The conditions were as for Fig. 1. (*a*) InSS; (*b*) InSR.

Table 7. *Protein synthesis by InSS and InSR*

Values are given as μg./ml. of medium. Dihydrostreptomycin or chloramphenicol was present at concentrations of 111 μg./ml.

Culture	Time (min.)	Control	Dihydrostreptomycin	Chloramphenicol
InSS	30	21	10	0
	60	41	25	0
	90	61	40	0
	120	96	50	0
	180	136	50	0
InSR	30	60	80	0
	60	150	205	0
	90	270	280	0
	120	240	320	0
	180	380	390	0

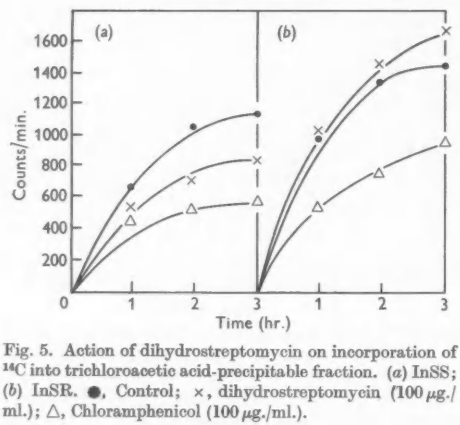

Fig. 5. Action of dihydrostreptomycin on incorporation of ^{14}C into trichloroacetic acid-precipitable fraction. (*a*) InSS; (*b*) InSR. ●, Control; ×, dihydrostreptomycin (100 μg./ml.); △, Chloramphenicol (100 μg./ml.).

toxic effect on *V. cholerae* by inhibiting oxygen uptake in the terminal respiration sequence in a manner somewhat similar to its action on *Escherichia coli* (Umbreit, 1949; Oginsky, Smith & Umbreit, 1949; Umbreit, Smith & Oginsky, 1951; Oginsky, 1953; Umbreit, 1953). Oxidation of the intermediates of the tricarboxylic acid cycle (excepting citrate, to which the cells are not permeable, and malate, the oxidation of which is not affected at all) is inhibited by the drug from the moment the substrate and enzyme are brought together. Experiments with intact cells indicate a multiplicity of steps in streptomycin inhibition; unequivocal evidence with cell-free extracts could, however, be provided only for the inactivation of succinic dehydrogenase and fumarase.

The inhibition data on oxidations by intact cells of *V. cholerae* fit in with observations on other bacteria, particularly acetate accumulation in sensitive organisms and the reversal of streptomycin inhibition by organic acids such as fumaric acid and pyruvic acid (see Pratt & Dufrenoy, 1949). The insensitivity of malate oxidation to streptomycin is a unique characteristic of *V. cholerae*. In an independent study of the metabolism of dihydrostreptomycin-sensitive, dihydrostreptomycin-resistant and dihydrostreptomycin-dependent strains of *Vibrio comma*, Farkas-Himsley (1957) found striking differences among the three strains in the rate of reduction of neo-tetrazolium in the presence of various substrates, except with malate.

The interaction of streptomycin and nucleic acids first demonstrated by Cohen (1946) has been suggested by Donovick, Bayan, Canales & Pansy (1948) as a possible mechanism of action of the drug. Peretz & Polglase (1956–57) showed that dihydrostreptomycin–nucleic acid complexes made from sonic extracts of drug-sensitive *E. coli* are not attacked by nucleases, in contrast with the ready susceptibility to enzyme action of similar complexes of dihydrostreptomycin with yeast ribonucleic acid and with thymus deoxyribonucleic acid. The non-availability of nucleotides or nucleic acid fragments implicated to function as the 'incorporation factors' (Gale, 1958) by the neutralization of nucleic acids by dihydrostreptomycin could lead to cessation of incorporation and possibly also of protein synthesis. That such a situation does not occur in *V. cholerae* is clear from the fact that dihydrostreptomycin–nucleic acid complexes of both InSS and InSR are equally attacked by nucleases. Thus although dihydrostreptomycin precipitates the nucleic acids of InSS, the latter are presumably still available for protein synthesis.

Protein synthesis and incorporation of labelled amino acid in InSS are both inhibited by dihydrostreptomycin although not to the same extent as by

chloramphenicol. It may be that this inhibition in *V. cholerae* by dihydrostreptomycin is secondary to a primary block at some other site. An aerobic organism such as *V. cholerae* derives its energy for synthetic processes, such as protein formation, by the terminal oxidation of carbon compounds. On the basis of the inhibition of two or more enzymes participating in the tricarboxylic acid cycle, it does not appear to be unreasonable to conclude that oxidative metabolism is the process primarily attacked by the drug in *V. cholerae*. A more rigorous proof of this could be provided only with isolated enzymes in reconstituted systems where the trapping of energy derived by oxidative breakdown of primary substrates could be demonstrated. This has not been possible in the present study owing to the presence in cell extracts of a highly active nucleotidase (Krishna Murti & Shrivastava, 1955*b*) insensitive to fluoride ions.

SUMMARY

1. Washed suspensions and extracts of a streptomycin-sensitive Inaba strain (InSS) of *Vibrio cholerae* and a streptomycin-resistant mutant (InSR) derived from it were used in manometric enzymic studies with a view to locating the site of action of dihydrostreptomycin.

2. The oxidation of acetate, succinate, fumarate, L-aspartate, DL-serine, L-cysteine, glycine, DL-threonine, DL-histidine, glucose, mannose and maltose were inhibited appreciably by concentrations of dihydrostreptomycin that prevented the growth of InSS. Malate oxidation by InSS was, however, not affected by the drug.

3. Oxygen uptake by InSR on these substrates was unaffected by dihydrostreptomycin even at a concentration 100 times that which inhibited InSS.

4. Dihydrostreptomycin also inhibited partially purified preparations of succinic dehydrogenase and fumarase made from InSS but not the corresponding enzymes derived from InSR.

5. Dihydrostreptomycin interfered with protein synthesis and incorporation of ^{14}C from [2-^{14}C]-glycine into the trichloroacetic acid-precipitable fraction of InSS and not into that of InSR.

6. Dihydrostreptomycin–nucleic acid complexes isolated from InSS and InSR are equally susceptible to the action of added nucleases.

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A Study of the Effect of Particle-Bound γ-Glutamyltranspeptidase on the Product of Interaction of Fluoropyruvate with Glutathione

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Fluoropyruvate has been shown to interact with thiol compounds (Avi-Dor & Mager, 1956*a*; Peters & Hall, 1957). Its inhibitory action on the respiration of mitochondria (Avi-Dor & Mager, 1956*b*; Chari-Bitron & Avi-Dor, 1958) and of tissue cells (Traub & Ginzburg, 1959) is presumably due to combination with thiol groups. We have observed that, during an incubation of the product formed between glutathione and fluoropyruvate with a rat-kidney homogenate, glutamate is liberated. Concomitantly the extinction in the ultraviolet region increases greatly. The change in the spectrum allows the kinetics of the reaction to be followed in the spectrophotometer. It can be demonstrated that the enzyme responsible for the reaction is in the microsomal fraction. The pattern of the reaction of the microsome-bound enzyme suggests that it is identical with γ-glutamyltranspeptidase, which is known to transfer the γ-glutamyl moiety of glutathione to L-amino acids, or, in their absence, to water (for references see Revel & Ball, 1959).

EXPERIMENTAL AND METHODS

Materials. Fluoropyruvic acid was synthesized as described by Blank, Mager & Bergmann (1955). The amino acids and peptides used were the products of the following firms: Glycylglycine, glycylglycylglycine and L-*S*-ethylcysteine of Mann Research Lab.; DL-α-amino-*n*-butyric acid, DL-*p*-fluorophenylalanine, L-ethionine and D-methionine of the California Foundation; the other amino acids of Schwarz Lab. Inc. All other chemicals used were of A. R. grade.

Preparation of rat-kidney microsomes. The cortex of the kidneys from rats (male and female) of 200–230 g. weight was homogenized with 10 (v/w) parts of an ice-cold 0·25 M-sucrose solution and the microsomal fraction separated as described by Schneider (1948). The microsomes were washed twice with water and resuspended in water. The final suspension, which contained approx. 20 mg. of protein/ml., was frozen and kept at −15°. No loss in the enzymic activity was noted during a storage of 6 weeks.

Preparation of the product of the reaction of thiols and fluoropyruvic acid. A mixture containing 50 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris buffer), pH 8·2, the respective thiol compound (3 mM) and 10 mM-fluoropyruvic acid was incubated at 37° for 20 min. Analysis by methods described previously (Avi-Dor & Mager, 1956*a*) has shown that the free SH groups of all the thiol compounds used disappeared at the end of incubation, with the liberation of an equivalent amount of hydrofluoric acid. While the structures of the products of interaction are not elucidated they are designated below as glutathione pyruvate, cysteinylglycine pyruvate and cysteine pyruvate respectively.

Determination of enzyme activity. During the action of the enzyme on glutathione pyruvate the largest increase in the extinction, *E*, occurred at 300 mμ. Thus, by recording the change in the extinction at this wavelength, the time curve of the reaction could be followed. Four Beckman silica cells of 1 cm. light-path were used; all cells (1, 2, 3 and 4) contained 50 mM-tris buffer (pH 8·2), the acceptor amino acid at the concentration required and water to 3·0 ml. Cells 1 and 2 also contained glutathione pyruvate (as a rule 0·3 mM). Before starting the experiment, the microsomes or other cell fractions were added to cell 3 in a volume of 0·02–0·03 ml. and the same volume of water was added to cells 2 and 4. The reaction was initiated by adding the cell fraction to be tested to cell 1. The amount of the particles tested for enzymic activity was chosen in such a way that the sample added to cell 1 could cause an increase in $E_{300\ m\mu}$ of 0·05–0·10 unit/min. when measured against cell 3 as the control. Measurements were carried out in the Beckman DU spectrophotometer at time-intervals of 15 or 30 sec. If not stated otherwise, measurements were carried out at room temp. (26–28°).

Calculation. When the reaction continued until no further change in $E_{300\ m\mu}$ was noted, the final level of

Fig. 1. Final extent of hydrolysis of glutathione pyruvate in the hydrolytic reaction (○) and in the transpeptidase reaction (●). Microsomes in amount equivalent to 120 μg. of protein/ml. were present. In the transpeptidase assay, a concentration of 10 mM-glycylglycine was present. Incubation was carried out for 30 min. at 37°. The microsomes were removed after the incubation by centrifuging and the extent of the hydrolysis was determined as described in the Experimental section.

absorbancy increased proportionally with the glutathione concentration in a wide concentration range. The same final state was reached whether the enzymic reaction had been carried out in the presence or in the absence of glycylglycine (Fig. 1). The increment in $E_{300\,m\mu}$ was 5·2 for a mM-glutathione pyruvate solution. Hence, without making any assumptions on the nature of the chromogenic hydrolysed product, the amount of glutathione pyruvate disappearing can be calculated from the expression:

$$(E_{300\,m\mu}\ \text{cell}\ 1/\text{cell}\ 3 - E_{300\,m\mu}\ \text{cell}\ 2/\text{cell}\ 4)/5{\cdot}2.$$

It is, however, shown below that at least 90% of the increase in $E_{300\,m\mu}$ is due to formation of cysteinylglycine pyruvate.

Interference. The effects of potassium cyanide, reducing agents (Avi-Dor & Mager, 1956*a*), borate (Avi-Dor & Lipkin, 1958) and bivalent metals (Avi-Dor, 1959) have been described before. During the present investigation 0·6 mM-chloramphenicol or 0·06 mM-tetracyclin (achromycin) depressed the extinction of cysteinylglycine pyruvate or cysteine pyruvate at the peak by 50%.

Definitions. Hydrolytic activity (V_H) is the number of μmoles of glutathione pyruvate hydrolysed/min./mg. of protein, in the absence of added acceptor amino acid. Transpeptidase activity (V_{TP}) is defined similarly, but in the presence of an acceptor amino acid.

The acceleration (*Acc*) caused by an acceptor is given by V_{TP}/V_H. The maximum acceleration ($Acc_{max.}$) is that caused by an infinitely high concentration of the acceptor.

Rates were calculated always from the linear part of the activity–time curve.

Protein. This was estimated by the method of Lowry, Rosenbrough, Farr & Randall (1951).

RESULTS

Identification of the products formed during the action of microsomes on glutathione pyruvate

Paper chromatography. With water-saturated phenol containing 0·002% of 8-hydroxyquinoline as the solvent in descending chromatograms run for 8 hr. at 25° untreated glutathione pyruvate remained near the starting line. The spot showed no fluorescence on illumination with an ultraviolet-light source and gave a positive reaction with ninhydrin. When the glutathione pyruvate had been incubated with microsomes until the increase in E reached its maximum, a fluorescent, ninhydrin-negative spot remained near the starting line and a strongly ninhydrin-positive spot appeared, possessing the same R_F (approx. 0·22) as glutamate run simultaneously. After prolonged incubation with the microsomes a weak spot corresponding to glycine could be detected. When L-methionine in an equivalent concentration to glutathione pyruvate was included in the incubation mixture the chromatogram revealed, besides the glutamate spot and the methionine spot (R_F 0·80), an additional spot (R_F 0·50). When this compound was extracted from the paper with water, hydrolysed in 6N-HCl for 14 hr. in a closed vessel at 110°, evaporated to dryness, redissolved in water and rechromatographed, it yielded two spots identified as methionine and glutamate. Thus, in the presence of the acceptor amino acid, the microsomal enzyme transferred the γ-glutamyl moiety to L-methionine and a dipeptide, presumably γ-glutamylmethionine, was formed.

Paper electrophoresis. On electrophoretic fractionation, glutathione pyruvate (Whatman no. 1 paper, 0·2M-acetate buffer of pH 4·6, 26v/cm., 2·5 hr., 20°) produced a single line (non-fluorescent, ninhydrin-positive, approx. 7·5 cm. from the starting line in the direction of the cathode). Cysteinylglycine pyruvate separated clearly from this and was fluorescent, ninhydrin-negative, approx. 9·5 cm. from the starting line. Cysteine pyruvate produced a yellow, visible line (weakly ninhydrin-positive, yellow fluorescent, approx. distance from starting line 4 cm.) and a second line which ran only slightly faster than cysteinylglycine pyruvate (fluorescent, ninhydrin-negative, approx. distance from starting line 10·0 cm.). The product of incubation of glutathione pyruvate with microsomes showed a fluorescent, ninhydrin-negative line corresponding exactly to that of cysteinylglycine pyruvate and a ninhydrin-positive line running just behind the glutathione pyruvate reference and corresponding to glutamate (identified also after elution, by paper chromatography). When the line given by the product of the enzyme action, corresponding to cysteinylglycine pyruvate, was eluted, hydrolysed and rechromatographed (with water-saturated phenol as solvent above), the liberation of glycine could be demonstrated.

Spectrophotometry. Further support for the assumption that the chromogenic compound formed during the action of microsomes on glutathione pyruvate is cysteinylglycine pyruvate came from an investigation of the spectra of the thiol–pyruvate compounds. Avi-Dor & Mager (1956*a*) reported that only those β-aminothiols which possess an unsubstituted amino group show high light-absorption in the ultraviolet. An increase in the extinction is, therefore, expected to occur when glutathione pyruvate is hydrolysed either to glutamate and cysteinylglycine pyruvate or to glutamate, glycine and cysteine pyruvate. Cysteine pyruvate and cysteinylglycine pyruvate have very similar spectra (Table 1) and the millimolar extinction of both compounds is 5·2 at 300 mμ. On the addition of 4 mM-zinc sulphate, however, the peak of cysteine pyruvate is strongly depressed while that of cysteinylglycine pyruvate remains unchanged. When the two compounds were mixed in various proportions each of them retained its properties in relation to the effect of the addition of Zn^{2+} ions. Even the presence of 10% cysteine pyruvate in cysteinylglycine pyruvate could be

Table 1. *Peaks of light extinction of thiol pyruvate compounds*

Incubation of glutathione pyruvate with microsomes was carried out as described for Fig. 1. When indicated 4 mM-zinc sulphate was added to the reaction mixture and the light absorption was measured 5 min. after the addition of the metal ions.

Compounds	$\lambda_{max.}$ (mμ)		$\epsilon_{mM}^{max.}$	
	No addition	Zn^{2+}	No addition	Zn^{2+}
Glutathione pyruvate				
Untreated	270	295	0·54	5·38
Incubated with microsomes	297	297	5·30	5·26
Cysteinylglycine pyruvate	297	297	5·35	5·39
Cysteine pyruvate	300	300	5·20	0·70

Table 2. *Distribution of the γ-glutamyltranspeptidase activity of rat-kidney cortex between various fractions of the cytoplasmic extract*

Temp. 28°. For the method of assay and the definition of the units used see Experimental section. The sum of the activity of the separated fractions was higher than that of the original cytoplasmic extract, namely for the hydrolytic reaction 160% and for the transpeptidase reaction 145%.

Fraction	Protein (% of total)	Hydrolytic activity (μmole/mg. of protein/min.)	Transpeptidase activity (μmole/mg. of protein/min.)
Cytoplasmic extract	100	0·03	0·45
Mitochondria	18	0·015	0·19
Microsomes	25	0·16	2·15
Supernatant	53	0·01	0·15
Microsomes (1 part) + mitochondria (1 part)	—	0·10	1·42
Microsomes (1 part) + supernatant	—	0·09	1·28

detected by the depression of *E*. The product of incubation of glutathione pyruvate with microsomes showed the same spectrum as cysteinylglycine pyruvate and was almost unaffected by the addition of zinc sulphate.

Distribution of γ-glutamyltranspeptidase activity in kidney homogenates

Transpeptidase and hydrolytic activity of various cell fractions isolated according to the method of Schneider (1948) were assayed by the standard procedure described under 'Methods'. The so-called nuclear fraction, which sedimented by using a centrifugal force of 700 *g* for 10 min., showed an activity varying from preparation to preparation. Since this fraction contains, besides nuclei, some damaged cells, it was suspected that the activity was due to this contamination. When a more homogeneous nuclear fraction was prepared from a sucrose–$CaCl_2$ homogenate (Hogeboom, Schneider & Striebich, 1952), the activity of the nuclear fraction was negligible. In the following experiments the sucrose homogenate, after the removal of the nuclear fraction (cytoplasmic extract), served as the starting material for the differential centrifuging. The data presented in Table 2 show average values from three experiments. In each experiment the homogenate was prepared from the kidneys of twelve rats. It can be seen that the highest activity is connected with the microsomal fraction. The small activity of the other fraction may be due to microsomal contamination. When microsomes were diluted with an equal amount of mitochondria or supernatant (based on the protein contents) and the dilution caused by the inert protein was taken into account, no significant change in the activity was observed.

Properties of the enzyme

Time course of the enzymic reaction. The time course of the reaction is shown in Fig. 2. In the absence of an added acceptor, a lag period is apparent. Glycylglycine, which accelerated the hydrolysis nearly 20 times, abolished the lag. Tests on other amino acids showed that their effect on the lag is related to their effectiveness as accelerators (acceptors). L-Methionine and L-glutamine shortened the lag, while glycine and L-glutamic acid had no effect.

Effect of the concentration of glutathione pyruvate. A double reciprocal plot of the glutathione pyruvate concentration against the velocity of the reaction (Fig. 3) shows that the affinity of the substrate to the enzyme is the same in the hydrolytic reaction as in the transpeptidation. The presence of the acceptor alters the maximum velocity but not Michaelis's constant (K_m 0·24 mM). When free glutathione (GSH) was used as the substrate instead of glutathione pyruvate, and the rate of hydrolysis was followed by determining the

amount of cysteinylglycine liberated (Avi-Dor & Mager, 1956*a*) K_m calculated from a Lineweaver–Burk graph was 0·5 mM.

Effect of the concentration of the enzyme and of pH. In the range 10–100 μg. of protein/ml. for the hydrolytic reaction and 0·8–8·0 μg. of protein/ml. for the transpeptidase activity, the velocity of the reaction was proportional to the amount of microsomes added. The optimum pH, in the presence as well as in the absence of acceptor, was about 8·15–8·30. The slope of the pH–activity curves on the acid side of the optimum depends on the pK of the amino group of the acceptor. Half-maximum activity was found with glycine (pK 9·60) at pH 7·75 and with glycylglycine (pK 8·15) at pH 6·90. These results closely resemble the findings of Kinoshita & Ball (1953) and Ball, Revel & Cooper (1956).

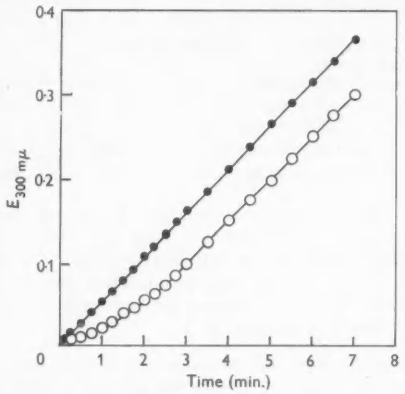

Fig. 2. Time course of the hydrolytic reaction (○) and of the transpeptidase activity (●). In the transpeptidase assay, 10 mM-glycylglycine was present and microsomes in an amount equivalent to 1·5 μg. of protein/ml. were used. To make the rate of the reaction of comparable magnitude microsomes equivalent to 30 μg. of protein/ml. were added in the hydrolytic reaction. In other respects the conditions of the reaction were as described in the Experimental section.

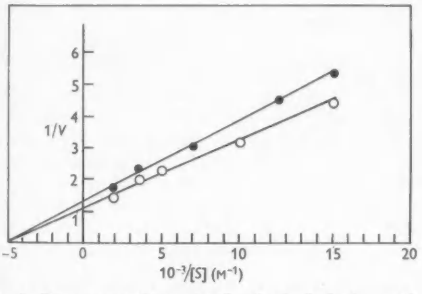

Fig. 3. Lineweaver–Burk plot for the hydrolytic reaction (○) and for the transpeptidation (●). The concentration of glutathione pyruvate was varied as indicated. In other respects conditions were as in Fig. 2.

Comparative activity of various acceptors. The comparative reactivity of various amino acids with GSH has been investigated in the past mostly by using an arbitrarily fixed concentration of the acceptor. However, the slope of the concentration–activity curves differs for each substance, and at higher concentrations a decline in the activity has been noted in some cases (Fodor, Miller & Waelsch, 1953; Hird & Springell, 1954; Revel & Ball, 1959). A comparison of the activities at an arbitrarily fixed level is therefore not strictly valid. The simplicity of the spectrophotometric method made it feasible to run concentration–activity curves for each acceptor and (from a double reciprocal plot, as exemplified in Fig. 4) to calculate the concentration causing half-maximum acceleration ($K_{Acc.}$) and maximum acceleration ($Acc_{max.}$) attainable at an infinitely high concentration of the substance. $K_{Acc.}$ and $Acc_{max.}$ values for a number of acceptors are listed in Table 3. D-Alanine, D-glutamic acid and D-methionine neither accelerated the hydrolytic reaction nor inhibited the transpeptidase activity with the respective L-isomer as the acceptor.

DISCUSSION

The pattern of the reaction of the microsome-bound enzyme obtained from rat-kidney homogenates suggests that it is identical with the so-

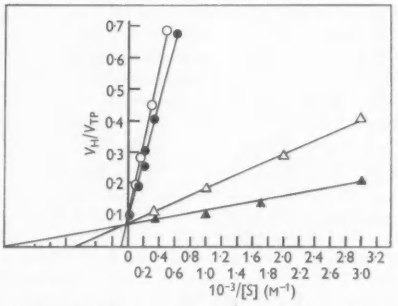

Fig. 4. Effect of the concentration of the acceptor on the extent of acceleration of the hydrolytic reaction: double reciprocal graph of the acceleration (V_{TP}/V_H) against the concentration of the acceptor (S). ○, Glycine; ●, ethylglycine; △, glycylglycylglycine; ▲, glycylglycine. Standard conditions of assay. The negative reciprocal value of the point of intersection of the graphs with the abscissa gives $K_{Acc.}$; the reciprocal value of the intersection with the ordinate gives $Acc_{max.}$.

Table 3. *Comparative reactivity of various acceptors*

The condition of the experiment and the method of the calculation of $K_{Acc.}$ and $Acc_{max.}$ were as described for Fig. 4. When DL-amino acids were used, the figure given is related to the concentration of the L-isomer.

Acceptor	$10^3K_{Acc.}$	$Acc_{max.}$	Acceptor	$10^3K_{Acc.}$	$Acc_{max.}$
Glycine	12·0	10·0	DL-α-Amino-*n*-butyric acid	4·0	9·0
Glycine ethyl ester	10·0	10·0	DL-Norvaline	5·0	9·0
Glycylglycine	0·7	18·0	DL-Norleucine	5·5	9·0
Glycylglycylglycine	1·4	13·0	L-Leucine	6·5	5·0
L-Alanine	2·4	9·0	DL-Isoleucine	10·0	2·5
L-Serine	5·0	7·0	L-Glutamic acid	4·3	4·5
Azaserine	—	1·0	L-Aspartic acid	—	1·0
L-*S*-Ethylcysteine	2·5	12·5	L-Glutamine	1·2	10·0
DL-Phenylalanine	5·0	6·0	L-Asparagine	2·2	5·0
DL-*p*-Fluorophenylalanine	5·0	4·0	L-Methionine	2·0	10·0
			L-Ethionine	2·0	10·0

called γ-glutamyltranspeptidase. Glutathione pyruvate as a substrate for the enzyme has a K_m of about the same magnitude as free glutathione. The products of hydrolysis are the same, except that for the former, instead of the free thiol compound, the thiol pyruvate derivative of high extinction is formed. The possibility of following the reaction spectrophotometrically greatly facilitates its quantitative study. There is no close correlation between the concentration of the acceptor causing half-maximum acceleration and the extent of the activation induced. This is especially striking if one compares the respective values for glycine ($K_{Acc.}$ 12; $Acc_{max.}$ 10) on the one hand and for its next homologue L-alanine ($K_{Acc.}$ 2·4, $Acc_{max.}$ 9) on the other. The effects due to branching of the carbon chain, length of the peptide chain, presence of an additional carboxyl group and stereochemistry were the same as those found by other investigators and will not be discussed.

The initial acceleration of the hydrolytic reaction could be the result of the accumulation of a substance derived from glutathione. This substance could serve, instead of the water, as the primary acceptor for the glutamyl moiety in the subsequent quicker steady phase of the reaction. The abolition of the lag by glycylglycine is in accordance with such a view. However, the addition of possible products of hydrolysis of glutathione, e.g. glutamic acid or glycine, did not affect the duration of the lag. The effect of other cleavage products containing a thiol group (cysteine, cysteinylglycine) could not be tested by the present method. Hence the identity of the acceptor which is assumed to accumulate during the lag is still obscure. The physiological role of γ-glutamyltranspeptidase is still unknown. Hanes, Hird & Isherwood (1950) made the suggestion that it may be involved in some way in the synthesis of proteins. The finding that the enzymic activity is bound to the microsomal fraction is therefore of interest, since this fraction is considered to be the main site of protein synthesis.

SUMMARY

1. It is shown that a microsome-bound enzyme, which can be separated from rat-kidney homogenates, splits the compound formed by the interaction of glutathione and fluoropyruvic acid with a concomitant increase in the ultraviolet light absorption.

2. An analysis of the products and the pattern of the reaction suggest that the microsomal enzyme is identical with γ-glutamyltranspeptidase.

3. By a spectrophotometric method some quantitative aspects of this enzyme activity have been studied.

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The Metabolism of 6-Aminopenicillanic Acid and Related Compounds by *Penicillium chrysogenum* and its Possible Significance for Penicillin Biosynthesis

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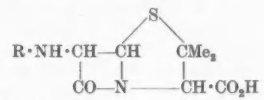
Several years ago it was reported that 6-aminopenicillanic acid (I) (then named penicin) is formed in cultures of *Penicillium chrysogenum* Q 176 when phenylacetate is omitted as side-chain precursor (Kato, 1953) or by enzymic hydrolysis of benzylpenicillin (II) by penicillin amidase (Sakaguchi & Murao, 1950). Until recently, however, compound (I) had not been properly characterized and its existence remained in doubt.



(I) R = H
(II) R = $C_6H_5 \cdot CH_2 \cdot CO$
(III) R = $HO_2C \cdot CH(NH_2) \cdot [CH_2]_3 \cdot CO$

The present search for (I) in mycelial extracts was prompted by the possibility that introduction of the side chain might represent the final step in penicillin biosynthesis (Arnstein, 1957) and by the report (Sheehan, 1958) that 6-aminopenicillanic acid had been synthesized, indicating that it was sufficiently stable to be capable of more than transitory existence. It was considered possible that conversion of (I) into (II) by reaction with phenylacetyl chloride (cf. Sheehan, 1958) might be followed by an increase in antibiotic activity, since acylations of this type are known to modify the biological activity of cephalosporin N (III) (Newton & Abraham, 1954).

Since at least some of the intermediates in penicillin biosynthesis may not be diffusible, as suggested by the work of Sermonti (1956) on heterokaryotic mutants (cf. Arnstein, 1957), it was decided to look for (I) in the mould mycelium rather than in the culture medium. Accordingly, we investigated the antibiotic activity of acetone extracts of mycelium and the effect of treating such extracts with phenylacetyl chloride. While this work was in progress, Batchelor, Doyle, Nayler & Rolinson (1959) published an account of their work on the isolation, characterization and properties of 6-aminopenicillanic acid (I), which had been isolated from the culture medium of *P. chrysogenum* grown in the absence of phenylacetate. Their work enabled us to identify by paper chromatography the major component of our 'phenylacetyl chloride-reacting substances' in mycelial extracts with (I). Subsequently, changes in the metabolism of these compounds in the presence and absence of phenylacetate were investigated by estimating the amounts present in both mycelium and culture medium and this aspect forms the main part of the present paper. Since, however, the increase in antibiotic activity obtained by reaction of crude extracts with phenylacetyl chloride was not correlated quantitatively with 6-aminopenicillanic acid, the term phenylacetyl chloride-reacting substances will be used throughout this paper.

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EXPERIMENTAL

Growth of organism and preparation of washed mycelium. *Penicillium chrysogenum*, WIS 51–20 F3, was obtained from Professor M. J. Johnson, Department of Biochemistry, College of Agriculture, University of Wisconsin. Subcultures were maintained on molasses–agar in medicinal flat bottles (area 36 in.^{2}). Mycelium were grown either in a fermenter (Arnstein & Grant, 1954) or in 250 ml. conical flasks on a rotary shaker (speed about 180 rev./min.) in the medium of Jarvis & Johnson (1950), usually as modified by Grau & Halliday (1958), without addition of phenylacetate unless specifically indicated.

In Expt. no. 1 (Table 1) spores from one flat bottle were used to inoculate four 100 ml. portions of unmodified medium (Jarvis & Johnson, 1950) and the flasks were incubated on the rotary shaker for 47 hr. The cultures were combined aseptically and one-half was used for preparing an acetone extract of the mycelium (see below). The remainder was divided into four 50 ml. portions, which were incubated further with or without 0·1% potassium phenylacetate as indicated in Table 1. The yields of mycelium (wet wt.) from these portions were 35 g. (47 hr.), 7·7 g. and 7·6 g. (72 hr.) and 5·2 g. and 8·2 g. (112 hr.). The dry wt. was assumed to be 10% of these values. In the absence of phenylacetate the antibiotic activity of the medium was 12·5, 60 and 50 i.u./ml. at 47, 72 and 112 hr. respectively; in the presence of phenylacetate the penicillin titres were 274 and 355 i.u./ml. at 72 and 112 hr. Expt. no. 2 was essentially similar except that the modified medium

was used, the original cultures were combined after 40 hr. growth and eight 50 ml. portions were incubated further. The wet wt. and dry wt. (in parentheses) of mycelium from successive 50 ml. samples (Table 1) were as follows (g.): 3·05 (0·266), 3·04 (0·344), 3·63 (0·429), 3·39 (0·432), 5·80 (0·547), 5·49 (0·530) and 6·19 (0·704). The ability of this mycelium to produce penicillin in the presence of phenylacetate was tested in the washed-mycelium experiment WM1 described in Table 2. It was found that after 60 hr. this fermentation was exceptional in that the penicillin-producing capacity of the mycelium fell precipitously. For Expt. no. 3 the organism was grown in a fermenter in 1·5 l. of the modified medium without phenylacetate, and portions (21–45 ml.) were withdrawn aseptically at the stated intervals. At 48 hr., three 40 ml. samples were transferred to conical flasks and incubated with 0·1% potassium phenylacetate on the rotary shaker for 19 hr. The volumes of culture medium, yields of mycelium (g. dry wt. in parentheses) and antibiotic activity of the medium from successive samples (Table 1) were as follows: 25 ml., 0·350 g. (0·034), $< 0·6$ i.u./ml.; 45 ml., 3·32 g. (0·274), 17 i.u./ml.; 13·5 ml., 2·42 g. (0·257), 25 i.u./ml.; 21 ml., 2·34 g. (0·181), 28 i.u./ml.; 1320 ml. (remaining contents of fermenter at 64 hr.), 180·0 g. (18·7), 32 i.u./ml.; 110 ml. (cultures incubated with phenylacetate from 48–67 hr.), 12·3 g. (1·45), 266 i.u./ml.

For washed mycelium experiments, portions of mycelium (0·5 g. or 1·0 g.) that had been harvested by filtration and washed with water or 0·01 M-potassium phosphate buffer, pH 7 (Halliday & Arnstein, 1956), were incubated in 100 ml. conical flasks containing 0·01 M-potassium phosphate buffer (1·36 g. of KH_2PO_4 and 0·40 g. of KOH/l.), pH 7 (10 vol.), with or without potassium phenylacetate (0·005 or 0·05%), on the rotary shaker at 24° for 1–3 hr.

Expt. WM1 was done with mycelium from Expt. no. 2 (Table 1). Duplicate flasks, each containing 0·5 g. of mycelium, were incubated with 5 ml. of medium for 1 hr. After bioassay of the medium (individual flasks differed from the mean, which is given in Table 2, by less than 5%), the mycelia were combined and extracted with acetone for estimation of antibiotic activity and phenylacetyl chloride-reacting material. Other details are given in Tables 1 and 2.

For Expt. WM2, mycelium was grown on the rotary shaker for 62 hr., in the modified medium without phenylacetate. Portions (1 g. wet wt.; dry wt. 10%) were incubated with or without 0·005% of potassium phenylacetate for 0, 1, 2 and 3 hr. in duplicate flasks. Acetone extracts were prepared (see below) from the separated mycelium without further washing it.

Preparation of acetone extracts and reaction with phenylacetyl chloride. Portions of washed mycelium were extracted by stirring in cold acetone (5 ml./g. wet wt.). After they had stood for at least 15 min. at 2°, centrifuging yielded a supernatant solution which contained approx. 80% of the total detectable phenylacetyl chloride-reacting material in the mycelium. Portions of the fermentation medium were diluted with acetone (2–4 vol.) before phenylacetylation.

The acetone extract of mycelium or medium was made up to 2·5 ml. with acetone and freshly prepared 0·5 M-$NaHCO_3$ (0·08 ml.), bromothymol blue indicator solution (British Drug Houses Ltd.; 0·02 ml.) and 0·1 M-potassium phosphate buffer, pH 7–7·5 (0·2–0·4 ml.), were added to bring the final volume of water, including that in the sample, to 0·5–0·7 ml., the ratio of water to acetone being approx. 1:3.

Duplicate or triplicate tubes were prepared for each sample, a set containing sodium benzylpenicillin (4–10 units, potency 1670 i.u./mg.; Glaxo Laboratories Ltd.) was included as internal standards and all tubes were kept stoppered in an ice bath.

To one set was added 2·4% (v/v) of conc. HCl in acetone (0·15 ml.) in order to determine the effect of HCl liberated during reaction of the acid chloride; the duplicate sets received, with shaking, 2% (v/v) of phenylacetyl chloride in acetone (0·15 ml., 45 m-equiv.) prepared just before use. All tubes were left at 2°, with occasional shaking, for 20–25 min. Ether (4 vol.) was then added and, after mixing, the aqueous layer was allowed to separate and was frozen by cooling the tubes in solid CO_2. The ether–acetone supernatant layer, which contained unreacted phenylacetyl chloride, was decanted and the aqueous solutions were bioassayed for penicillin activity.

In the second washed-mycelium experiment (Expt. WM2) phenoxyacetyl chloride was used instead of phenylacetyl chloride. The mycelium (1 g. wet wt.) was extracted with acetone (5 ml.) at 0° and a portion of the extract (2 ml.) was added to 0·5 M-$NaHCO_3$ (0·25 ml.), 0·1 M-potassium phosphate buffer, pH 7 (0·25 ml.), and 10% (v/v) phenoxyacetyl chloride in acetone (0·1 ml.). After 1 hr. at 0°, the mixture was treated as described above and assayed for antibiotic activity. Control samples of the same extracts were treated identically except that phenoxyacetyl chloride was omitted. Incubation medium was bioassayed directly after filtration and suitable dilution where necessary. Portions (5 ml.) were similarly acylated with phenoxyacetyl chloride (0·1 ml. of the 10% solution in acetone) in the presence of 0·5 M-$NaHCO_3$ (0·5 ml.) and 0·1 M-phosphate buffer (0·5 ml.). A solution of sodium benzylpenicillin (5 ml., 103·5 i.u.) was treated in the same way as a control; the recovery of antibiotic activity was 77% and the results of this experiment have been corrected accordingly (see below).

Bioassay and calculation of results. Bioassay of the aqueous solutions from the phenylacetylation procedure on agar plates seeded with *Bacillus subtilis* by the method of Humphrey & Lightbown (1952) yielded results which were corrected for the percentage recovery of penicillin in the internal standards (average recovery in 28 cases was 89%) and are expressed as units/ml. of medium or units/g. wet or dry wt. of mycelium. Dry weights were obtained by extracting the mycelial residue a second and third time with acetone and drying at 110°; the dry wt. varied from 8·5 to 12% of the wet wt. Suitable controls, such as buffer solutions, fermentation medium obtained from cultures before the beginning of the penicillin-producing phase, and sodium benzylpenicilloate (equivalent to 30 units of sodium benzylpenicillin), showed no antibiotic activity before or after phenylacetylation. There was no significant difference (< 4%) in activity between standard penicillin solutions treated with HCl and those treated with phenylacetyl chloride.

Chromatography. Paper chromatograms were always run by the ascending technique at 2° with the upper layer of butan-1-ol–ethanol–water (4:1:5, by vol.) (Batchelor *et al.* 1959) and propan-1-ol–water (7:3, v/v) with 1 cm. strips of Whatman no. 3 MM paper. After drying, the paper was cut in half longitudinally and one part was treated with

0·5 M-$NaHCO_3$ and 2% (v/v) phenylacetyl chloride in acetone. The two halves were then developed on an agar plate seeded with *B. subtilis*.

With other solvents, descending chromatography at room temperature was used, the composition (by vol.) of the various mixtures being as follows: (*a*) butan-1-ol–acetic acid–water (63:10:27); (*b*) pyridine–water (4:1); (*c*) pentan-1-ol–pyridine–water (7:7:6).

Reaction of an acetone extract of mycelium with labelled phenylacetyl chloride. [*carboxy*-^{14}C]Phenylacetic acid (16·6 mg., 150 μc, 1·23 mc/m-mole; obtained from The Radiochemical Centre, Amersham, Bucks) was warmed at 100° with an excess of thionyl chloride (0·30 ml.) for 30 min. After cooling to room temperature, excess of thionyl chloride was removed *in vacuo* (water pump). The residue was dissolved in acetone (2 ml.), 0·1 M-phosphate buffer, pH 7 (2 ml.), was added followed by an acetone extract (16 ml.) prepared from 6·16 g. (wet wt.) of mycelium. Solid $NaHCO_3$ (125 mg.) was added and, as the pH was still slightly acid, more $NaHCO_3$ was added at intervals whilst the mixture was shaken at 2° for 30 min. Ether (50 ml.) was added, the solution was mixed and the aqueous layer was frozen by cooling in solid CO_2. The ether layer was separated and the aqueous layer was re-extracted in a similar way with ether (30 ml.). The combined ether layers were re-extracted with 0·1 M-phosphate buffer, pH 7 (5 ml.). Bioassay showed that the first aqueous solution contained 17·3 i.u./ml., whereas the second contained only 0·2 i.u. of penicillin/ml. The total amount of penicillin activity was approx. 38 units (equivalent to 23 μg. of sodium benzylpenicillin). Carrier sodium benzylpenicillin (100 mg.) was added, the penicillin was extracted with ether at pH 2 and converted into the *N*-ethylpiperidine salt, which was recrystallized four times from dry chloroform–acetone (1:4, v/v). This product was degraded to penilloaldehyde (Arnstein & Clubb, 1957) which was isolated as the 2:4-dinitrophenylhydrazone. After crystallization from ethanol it was chromatographed first on paper with pentan-1-ol–pyridine–water as solvent and then on an alumina column with ethyl acetate as eluent.

The mother liquors from the first recrystallization of the *N*-ethylpiperidine salt of benzylpenicillin were chromatographed (Leigh, 1949) on a column (38 cm. × 1·2 cm. diameter) of silica gel (10 g.) prepared by suspending the silica gel in ethyl acetate (50 ml.) and adding water (7 ml.) and *N*-ethylpiperidine (0·5 ml.). Ethyl acetate containing 0·1% of *N*-ethylpiperidine was used as eluent and 5 ml. fractions were collected.

Radioactivity determinations. Measurements were made with a thin end-window Geiger–Müller tube (type EHM 2/s, General Electric Co. Ltd.) with samples of infinite thickness on 1 cm.^{2} polythene or 0·3 cm.^{2} Perspex disks (Popják, 1950).

RESULTS

Evidence for the presence of 6-aminopenicillanic acid in phenylacetyl chloride-reacting material

When extracts of mycelia or media from fermentations from which phenylacetate had been omitted were treated with phenylacetyl chloride at pH 7, a consistent increase in antibiotic activity was observed. This activity was sensitive to penicillinase and was presumed to be due to a penicillin. After the paper by Batchelor *et al.* (1959) had been published, paper chromatography by their method revealed the presence in such extracts of a major component which showed antibacterial activity after phenylacetylation. This compound has R_F 0·14 in butanol–ethanol–water, which is identical with that reported by Batchelor *et al.* (1959) for 6-aminopenicillanic acid (I). The R_F values of this substance in other solvents are: 0·50 in propanol–water, 0·34 in butanol–acetic acid–water, 0·60 in pyridine–water and 0·24 in pentan-1-ol–pyridine–water. In addition to this major component, varying amounts of other compounds have been detected in any one mycelial extract. These compounds had R_F values in butanol–ethanol–water of 0–0·05, 0·26 and 0·09.

The material before phenylacetylation was not extractable by ether at pH 2, but, like the product, was labile to alkali. The penicillinase-sensitive, major product of phenylacetylation was extractable with ether at acid pH and its behaviour on paper chromatography in butanol–ethanol–water and propanol–water was identical with that of benzylpenicillin.

In the experiment with [^{14}C]phenylacetyl chloride, the product (23 μg. of benzylpenicillin by bioassay) was diluted with carrier and isolated as the *N*-ethylpiperidine salt, which had specific radioactivies of 3·23, 2·80, 2·96 and 3·02 μc/g. upon successive recrystallizations. The average specific activity of this product (3·00 μc/m-mole) was, however, considerably greater than that calculated from the original specific radioactivity of the labelled phenylacetyl chloride, the yield of penicillin after phenylacetylation and the amount of carrier (0·28 μc/mole). The penicillin was degraded to penilloaldehyde, which was converted into the 2:4-dinitrophenylhydrazone. After recrystallization from ethanol, 10 mg. with a specific radioactivity of 2·21 μc/g. (0·790 μc/mole) was obtained. The colour and radioactivity of the dinitrophenylhydrazone chromatographed as a single substance on paper and on alumina and the material obtained after these procedures (4 mg.) had almost the same specific radioactivity (1·87 μc/g., 0·67 μc/m-mole) as before. The difference in radioactivity of penicillin and the penilloaldehyde 2:4-dinitrophenylhydrazone obtained by degradation suggested the presence of a highly radioactive impurity in the penicillin despite the constant specific radioactivity.

Chromatography of the concentrated mother liquors from the first recrystallization of the *N*-ethylpiperidine salt on silica gel revealed a major peak of radioactivity (0·5 μc) associated with antibiotic activity in fractions nos. 37–100. When chromatographed on paper with butanol–ethanol–water or propanol–water as solvents, this material

Table 1. *Antibiotic activity in mycelial extracts of* Penicillium chrysogenum *before and after reaction with phenylacetyl chloride*

Mycelium was harvested at the stated times and extracts were prepared and bioassayed as described in the Experimental section. For details of fermentations see text.

Expt. no.	Time (hr.)	Phenylacetate present (+) or absent (−)	Antibiotic activity in mycelium: Before reaction with phenylacetyl chloride (i.u./g. dry wt.)	Increase after reaction with phenylacetyl chloride (i.u./g. dry wt.)	Increase after reaction with phenylacetyl chloride (%)
1	47	−	29·6	91·8	310
	72	−	89·0	61·3	69
	72	+	28·0	20·0	72
	112	−	66·8	21·2	32
	112	+	54·5	2·0	4
2	40	−	37·0	141	381
	46·5	−	67·8	195	228
	56·5	−	81·0	234	289
	56·5	−	63·3	239	377
	61	−	73·0	403	552
	61	−	77·4	340	434
	68	−	53·5	256	478
3	26	−	⩽10	⩽5	—
	43	−	45·3	108	238
	48	−	99·1	104	105
	55	−	66·4	276	415
	64	−	35·0	273	780
	67	+	51·3	4·3	8

Table 2. *Effect of incubating washed mycelium with or without phenylacetate on the amount of mycelial phenylacetyl chloride-reacting material (Expt.* WM 1)

Portions (0·5 g. wet wt.) of washed mycelium, obtained from Expt. no. 2 (Table 1) and harvested at various times, were incubated with or without phenylacetate in duplicate flasks. The amount of phenylacetyl chloride-reacting substances in the mycelium was determined by measuring the increase in antibiotic activity after the mycelial extracts had reacted with phenylacetyl chloride as described in the Experimental section. Zero-time values have been calculated from the results given in Table 1. For experimental details of the fermentation see text. All results are expressed on the basis of 10 ml. of medium and 1 g. (wet wt.) of mycelium.

Age of mycelium (hr.)	Incubation conditions: Time (min.)	Potassium phenylacetate added (%)	Mycelial dry wt. (mg.)	Antibiotic activity (i.u.): In medium	In mycelium: Initial	In mycelium: Increase after phenylacetylation
46·5	0	—	110	—	7·5	21·5
	60	0·05	102	206	12·5	6·9
56·5	0	—	118	—	9·6	27·6
		—	127	—	8·0	30·4
	60	0·05	115	142	10·8	9·4
		0·05	120	107	12·7	9·2
61	0	—	94	—	6·9	37·9
		—	97	—	7·5	33·0
	60	None	104	3·0	6·8	27·2
		None	103	None	6·8	23·4
	60	0·05	96	112	7·3	12·0
		0·05	93	102	10·2	10·9
68	0	—	112	—	6·0	28·6
	60	None	113	None	7·5	17·5
		0·05	109	None	9·6	6·8

behaved identically with benzylpenicillin. Two radioactive contaminants appeared in earlier fractions: one was phenylacetic acid of high specific radioactivity (total approx. 2·6 μc in fractions nos. 9–25), the other was an unknown compound which was eluted in fractions nos. 7 and 8 and contained both antibiotic activity and radioactivity (approx. 0·05 μc), as shown by coincidence on a two-dimensional chromatogram (butanol–ethanol–water, R_F 0·93, and propanol–water, R_F 0·87). It was clearly different from benzylpenicillin (R_F values 0·48 and 0·78 respectively).

Distribution of phenylacetyl chloride-reacting material in mycelium and culture medium and changes in its concentration during penicillin production

Although a small amount of phenylacetyl chloride-reacting material was observed in mycelial extracts from *Penicillium chrysogenum* grown for 72–118 hr. under the usual conditions with phenylacetate, with penicillin titres of 200–300 units/ml. a great deal more of these compounds was found in preparations grown for shorter times in the absence of phenylacetate (Table 1). Under these conditions some increase in penicillin titre after phenylacetylation was also found in the medium, representing a larger total amount, and sometimes also a higher concentration, than in the mycelium. In the absence of phenylacetate as side-chain precursor very little antibiotic activity (max. 60 i.u./ml. at 72 hr., Expt. no. 1) was found in the medium, whereas addition of phenylacetate gave titres of 270 and 360 i.u./ml. at 72 and 112 hr. respectively. The increase in antibiotic activity after treatment of medium with phenylacetyl chloride was usually between 5 and 25 i.u./ml. with cultures grown without phenylacetate, but was not detectable when phenylacetate had been added. No significant antibiotic activity was found at 26 hr. in either medium or mycelium before or after treatment with phenylacetyl chloride.

The maximum concentration in the mycelium appears to occur at about 60 hr. of growth. This time corresponds to that reported by Grau & Halliday (1958) for maximum penicillin-producing

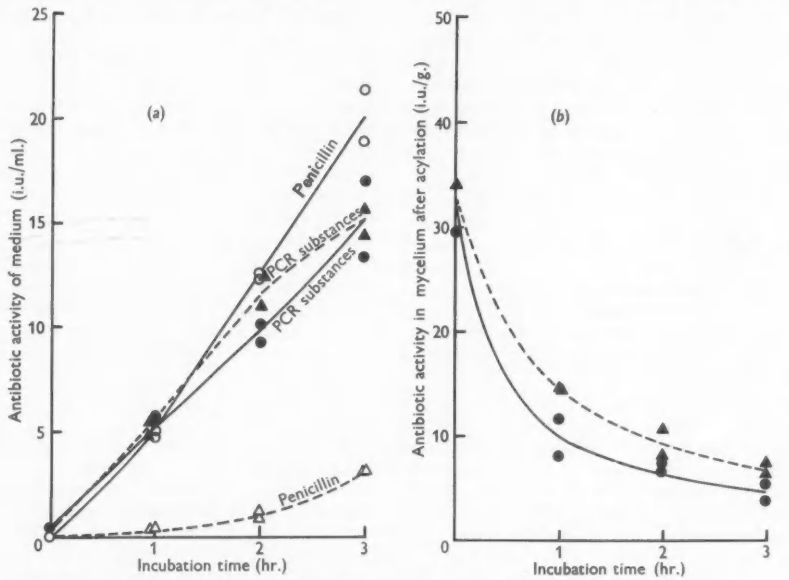

Fig. 1. Metabolism of phenylacetyl chloride-reacting substances by washed mycelium incubated with or without 0·005% potassium phenylacetate (Expt. WM2). Phenylacetyl chloride-reacting substances were estimated by the increase in antibiotic activity after acylation with phenoxyacetyl chloride (see text). (*a*) Activity of medium after incubation with (○) or without (△) phenylacetate and biosynthesis of phenylacetyl chloride-reacting substances in the presence (●) or absence (▲) of phenylacetate. (*b*) Antibiotic activity of mycelium, calculated/g. wet wt., after incubation with (●) or without (▲) phenylacetate. The values refer to the antibiotic activity after acylation; the activity of extracts before acylation was too low for accurate determination (< 2·5 i.u./g. wet wt.).

ability as measured in incubations of washed mycelium with 0·01% potassium phenylacetate.

It has also been found that storage of mycelium at 0° in oxygen for 24 hr. resulted in a marked increase of phenylacetyl chloride-reacting substances as well as of antibiotic activity before phenylacetylation (350% and 670% respectively). Storage at room temperature for 1 hr. led to similar increases (386% in phenylacetyl chloride-reacting substances and 564% in antibiotic activity before phenylacetylation).

Washed-mycelium experiments. Measurements of the changes in phenylacetyl chloride-reacting substrate when portions of washed mycelium were incubated with and without phenylacetate yielded results (Table 2 and Fig. 1) which were quantitatively somewhat variable. In all experiments, however, phenylacetyl chloride-reacting material in the mycelium decreased on incubation with phenylacetate even after only 1 hr. In three out of four experiments, this decrease was greater than that observed in the absence of phenylacetate, but in one case there was no significant difference.

Phenylacetyl chloride-reacting substances were also found in the medium and are biosynthesized by washed mycelium (Fig. 1*a*). Surprisingly, there was little difference in the amount of phenylacetyl chloride-reacting substances found in the medium on incubating with or without phenylacetate, even though the antibiotic activity before acylation, presumably due to benzylpenicillin, was much greater when phenylacetate was added. The decrease in phenylacetyl chloride-reacting substances in the mycelium (Fig. 1*b*) after incubating for 1 hr. in the presence of phenylacetate was similar to that observed in Expt. WM 1 (Table 2). Omission of phenylacetate had, however, a much less marked effect than previously, although the decrease in mycelial phenylacetyl chloride-reacting substances was consistently less in the absence of phenylacetate throughout the 3 hr. incubation period.

In all cases examined, the total amount of phenylacetyl chloride-reacting material found in the medium was greater than that lost from the mycelium. Since the amount of penicillin formed was approximately ten times the total decrease in phenylacetyl chloride-reacting substances, there must be a rapid turnover of phenylacetyl chloride-reacting material if it is a penicillin precursor.

DISCUSSION

A comparison of our results with those of Batchelor *et al.* (1959), which include the complete characterization of 6-aminopenicillanic acid (I), suggests that the main component of phenylacetyl chloride-reacting material in the mycelium of *Penicillium chrysogenum* is in fact (I), but that in addition there exists one or more perhaps closely related compounds which also give rise to increased antibiotic activity against *B. subtilis* on treatment with phenylacetyl chloride. Estimation of these compounds after chromatographic separation on paper indicates that their relative amounts vary; at most these compounds together may equal the amount of (I) although usually they appear to be present in less than half that quantity.

The results on the production of phenylacetyl chloride-reacting material in fermentations and washed mycelium incubations are not inconsistent with but do not prove a precursor–product relationship between (I) and penicillin (II). The most significant effect of phenylacetate seems to be an increase in penicillin production, whereas the amount of phenylacetyl chloride-reacting substances is not always correspondingly decreased (see Expt. WM 2). It is possible therefore that (I) and (II) are synthesized independently, but further work with labelled precursors would be required to decide this point. The presence of considerable amounts of δ-(α-aminoadipyl)cyst(e)inylvaline, the open-chain tripeptide corresponding to cephalosporin N (III), in the mycelium of *P. chrysogenum* (Arnstein & Morris, 1960) suggests that the common precursor of the penicillins and 6-aminopenicillanic acid may be cephalosporin N. Whether cephalosporin N is one of the minor constituents of phenylacetyl chloride-reacting material in our mycelial extracts remains to be investigated.

The observation that addition of phenylacetate results in increased penicillin titres but in either no change or a decrease in phenylacetyl chloride-reacting substances and hence presumably in (I) appears to favour one of the foregoing explanations rather than the possibility of a conversion of benzylpenicillin into 6-aminopenicillanic acid by the action of penicillin amidase (Sakaguchi & Murao, 1950).

SUMMARY

1. The mycelium of *Penicillium chrysogenum* WIS 51–20 F3 and the culture medium contain substances (phenylacetyl chloride-reacting substances) which give rise to enhanced antibiotic activity on acylation with phenylacetyl or phenoxyacetyl chloride.

2. One of these compounds appears to be identical with 6-aminopenicillanic acid.

3. The biosynthesis of phenylacetyl chloride-reacting substances has been studied with cultures or washed mycelium of *P. chrysogenum*.

4. Mycelia from cultures to which phenylacetate had been added contained little or no phenylacetyl chloride-reacting substances. Incubation of washed

mycelium in the presence of phenylacetate resulted in a greater decrease in mycelial phenylacetyl chloride-reacting substances than was found in the absence of phenylacetate.

5. No quantitative correlation was found, however, between the increased penicillin production resulting from the addition of phenylacetate and the decrease in phenylacetyl chloride-reacting substances.

6. The metabolic inter-relationship between 6-aminopenicillanic acid and penicillin is discussed.

We wish to thank Mr E. J. Toms for technical assistance.

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The Ultraviolet Fluorescence of Proteins in Neutral Solution

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The fluorescence characteristic of aqueous solutions of tyrosine, tryptophan and phenylalanine was described in detail by Teale & Weber (1957). The fluorescence of these aromatic amino acids when combined in the polypeptide chains of globular proteins is now reported and some structural implications of the results are discussed.

EXPERIMENTAL

Methods

Fluorescence spectra and fluorescence-excitation spectra. These were determined by means of the apparatus described by Teale & Weber (1957). Protein solutions contained in a quartz cell were irradiated by radiation of 2537 Å wavelength isolated from a low-pressure mercury arc by a combination of a Chance OX7 glass filter and *p*-nitrophenol solution in a silica cuvette. The fluorescence emitted at right angles to the direction of excitation was analysed by a Bausch and Lomb grating monochromator and detected by an E.M.I. 6255B quartz-window photomultiplier. The corrections for varying monochromator transmission and detector response with wavelength are described elsewhere (Teale & Weber, 1957). In certain cases a band of continuous radiation derived from a xenon-arc lamp by means of an aqueous solution of nickel sulphate and *p*-nitrophenol was used to excite over the whole absorption band of the protein.

Fluorescence-excitation spectra were obtained with a hydrogen- or xenon-arc lamp as the continuous source. A filter of Perspex 4 mm. thick or a Chance OX1 glass filter was used to separate exciting light from fluorescence. Cuvettes of extremely pure fused quartz (Thermal Syndicate Ltd., Wallsend, Northumberland, England) were used to reduce cell fluorescence over the 240 mμ excitation range. In the determination of both excitation and fluorescence spectra, experimental points were obtained at wavelength intervals of 5 mμ. Additional points were interpolated where the readings changed rapidly with wavelength.

Fluorescence quantum yields. These were measured by comparing the total emission from protein solutions with that of pure tyrosine or tryptophan solutions with the same excitation absorption. Absolute yields were then calculated by correction for differences in detector response to the fluorescence spectra, assuming the published values for the absolute quantum yields of tyrosine and tryptophan (Weber & Teale, 1957). For accurate measurements of the quantum yield in the wavelength region from 290 to 310 mμ, where the molecular extinction changes rapidly, monochromatic lines of the high-pressure mercury arc were employed to excite fluorescence.

Absorption spectra. These were determined with a Uvispek spectrophotometer at wavelength intervals of 5 mμ, or with an Optika recording spectrophotometer. The extinction coefficients of proteins at wavelengths where the absorption is small were obtained with concentrated

solutions and a dilution factor was applied. The light-scattering by protein solutions, indicated by finite absorption at wavelengths greater than 310 mμ, was reduced to a minimum by filtration or centrifuging.

Propane-1:2-diol–water mixtures of measured viscosity were used to determine the influence of viscosity on fluorescence, and solutions with a wide range of dielectric constants were obtained by using dioxan–water mixtures. Chymotrypsinogen was converted into the active enzyme by trypsin at pH 7·5 (molecular ratio 100:1).

Materials

Pure crystalline proteins for which there were reliable values of aromatic amino acid composition were selected for detailed investigation. Human-serum albumin, crystallized as the mercury dimer by the author, was a gift from Dr J. L. Oncley. Ovalbumin was prepared by the ammonium sulphate fractionation of egg albumin (Warner, 1954). Trypsin inhibitors and trypsin inactivated with di-*iso*propyl phosphorofluoridate were gifts from Dr N. M. Green. Fumarase was a gift from Dr V. Massey. The remaining proteins were available commercially as follows: bovine-serum albumin, haemoglobin, fibrinogen, γ-globulin, lysozyme and pepsin were supplied by Armour Laboratories; trypsin, chymotrypsinogen, chymotrypsin, pepsin, carboxypeptidase and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; trypsinogen and ribonuclease were products of Sigma Chemical Co., St Louis 13, Mo., U.S.A.; insulin was supplied by British Drug Houses Ltd., as was propane-1:2-diol having high transparency in the ultraviolet region.

Two attempts were made to remove tryptophan selectively from human-serum albumin. In one experiment the protein was irradiated in the presence of methylene blue with a low-pressure mercury arc in the hope of selectively photo-oxidizing tryptophan (Weil & Buchert, 1951). In another experiment the albumin was treated with chymotrypsin and carboxypeptidase simultaneously. As the molecule of albumin contains 17 tyrosine residues but only one tryptophan group, it was hoped that some partly degraded albumin molecules containing only tyrosine would be obtained.

RESULTS

Fluorescence characteristics

Table 1 contains the absolute quantum yields, calculated for equal total absorption at 280 mμ wavelengths, and the fluorescence-band maxima of 21 proteins, each in three different solvents. Table 2 lists the residues of tyrosine, tryptophan and phenylalanine occurring in each molecule of the proteins investigated, and also the fractional absorption of tryptophan at 280 mμ wavelength, calculated from the published molecular-extinction coefficients of tyrosine and tryptophan (Beaven & Holiday, 1952).

Fig. 1 shows typical excitation and fluorescence spectra of tryptophan-containing proteins and also of insulin. The excitation spectrum of human-serum albumin between 220 and 247 mμ is plotted on a changed ordinate scale. All the fluorescence-

Table 1. *Fluorescence yields* (q) *and wavelengths of the maxima of emission for proteins in water, 8*M*-urea and propane-1:2-diol*

The overall quantum yields at 280 mμ are denoted by q. The yields denoted by q_1 are calculated assuming that the fluorescence is due to the tryptophan residues alone and that there is no transfer of energy from tyrosine to tryptophan. $q_1 = q$ divided by the fraction of the total absorption due to tryptophan (see Table 2).

	Water			8 M-Urea			Propane-1:2-diol		
Protein	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)
Insulin	304	3·7	—	304	3·0	—	304	8·3	—
Zein	304	8·0*	—	—	—	—	304	8·0	—
Ribonuclease	304	1·7	—	304	1·7	—	304	3·2	—
Ovomucoid	304	1·2	—	304	1·2	—	304	2·0	—
Pancreatic trypsin inhibitor	—	0·0	—	—	0·0	—	—	0·0	—
Bovine γ-globulin	332	3·8	5·0	350	5·3	6·8	337	9·7	12·8
Lysozyme	341	6·0	6·5	350	4·1	4·4	343	6·0	6·45
Trypsin	332	8·1	12·6	350	13·8	21·4	335	14·1	21·8
Trypsinogen	332	8·7	13·4	350	14·0	22·0	335	14·0	21·6
Chymotrypsin	334	9·5	10·5	350	20·4	22·6	338	20·0	22·3
Chymotrypsinogen	331	7·2	8·0	350	20·0	22·2	336	15·8	17·6
Human-serum albumin	339	7·4	38·0	350	5·2	26·2	340	7·4	38·0
Bovine-serum albumin	342	15·2	47·5	350	7·4	23·0	343	15·2	47·5
Ovalbumin	332	12·1	20·9	340	13·1	22·4	336	18·1	31·0
Fumarase	335	9·0	—	—	—	—	—	—	—
Carboxypeptidase†	340	12·2	22·1	348	9·0	16·4	340	14·0	25·3
Pepsin	342	12·8	25·0	350	10·2	20·0	344	18·0	35·3
Fibrinogen	337	14·0	20·6	350	14·0	20·6	340	21·0	31·0
Edestin‡	328	11·8	23·0	350	12·0	23·5	—	—	—
Haemoglobin globin	335	10·0	14·3	348	8·0	11·4	—	—	—

* Ethanol–water (50:50). † 10% Lithium acetate solution. ‡ M-NaCl solution.

Table 2. *Distribution of the aromatic amino acids in proteins and the percentage absorption by tryptophan at 280 mμ wavelength*

Protein	Concn. (mM)	Phenylalanine residues	Tyrosine residues	Tryptophan	
				Residues	Percentage absorption
Lysozyme	15	3	3	8	93
Myoglobin	16·5	4	2	2	80
Trypsin/trypsinogen	24	4	9	4	64·5
Chymotrypsin/chymotrypsinogen	25·1	6	4	7	90
Carboxypeptidase	34·4	15	20	6	55
Pepsin	35	13	16	4	51
Ovalbumin	43	21	9	3	58
Edestin	50	16	12	3	51
Human-serum albumin	65	33	17	1	19·5
Bovine-serum albumin	65	24	18	2	32
Haemoglobin	66	30	11	6	70
Bovine globulin	156	28	18	14	76
Fibrinogen	450	28	30	16	68
Pancreatic trypsin inhibitor	7	5	5	0	0
Insulin	12	6	8	0	0
Ribonuclease	15	3	6	0	0
Ovomucoid	28	5	5	0	0
Zein	50	44	29	0	0

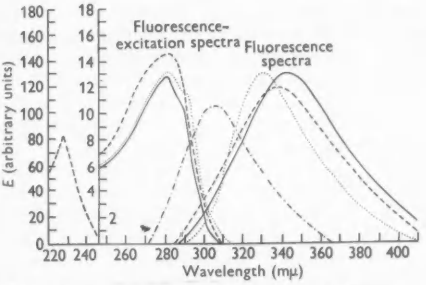

Fig. 1. Fluorescence-excitation spectra and fluorescence spectra of proteins in aqueous solutions. Chymotrypsin (···); insulin (—·—); human-serum albumin (– – –); pepsin (——). In the excitation spectrum of human-serum albumin between 220 mμ and 247 mμ, the ordinate scale of extinction is increased tenfold.

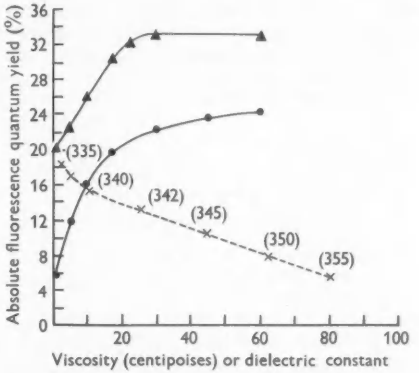

Fig. 2. Influence of the viscosity and dielectric constant of the medium on the fluorescence yields of tryptophan and glycyltryptophan in solution. ▲, Tryptophan yield against viscosity; ●, glycyltryptophan yield against viscosity; ×, glycyltryptophan yield against dielectric constant, with the wavelength (mμ; figures in parentheses) of the fluorescence maximum at selected points.

excitation spectra of proteins containing tryptophan were similar in profile and band maxima, and differed only at the edge of the long-wave-absorption band, where individual proteins showed variations in position accompanied by displacement of the fluorescence spectrum in the converse direction. These spectral shifts were similar to those shown by tryptophan and its derivatives in media of different polarizability, illustrated by Fig. 2, which shows the variation in absolute yield and fluorescence-band maximum of glycyltryptophan with the dielectric constant of the dioxan–water mixtures used as the solvent. Included in Fig. 2 are the variations of absolute yield of tryptophan and glycyltryptophan with solvent viscosity, with propane-1:2-diol–water mixtures.

The proteins listed in Table 1 did not show a fluorescence spectrum with a maximum at 280 mμ, as is given by phenylalanine. The table includes proteins such as human-serum albumin, in which the relative absorption by phenylalanine is large. Pure gelatin and the polypeptide polymyxin, in which phenylalanine is the only aromatic residue, exhibit a fluorescence similar to that of phenylalanine but with reduced quantum yield and showing vibrational structure. Phenylalanine itself shows vibrational structure in the absorption

spectrum but not in the fluorescence spectrum of aqueous solutions. The differences probably reflect the lower polarizability of the molecular environment of combined phenylalanine in gelatin and polymyxin, compared with that of the free amino acid in aqueous solution (Teale & Weber, 1957; F. W. J. Teale, unpublished results).

Proteins containing tyrosine but no tryptophan showed tyrosine fluorescence in low yield or were non-fluorescent. The fluorescence-excitation spectra of insulin and ribonuclease were similar in band position ($\lambda_{max.}$ 227 and 276 mμ) and profile to that of pure tyrosine in water ($\lambda_{max.}$ 224 and 275 mμ) with a slight displacement to longer wavelengths. In the 260 mμ region a contribution of phenylalanine excitation to tyrosine fluorescence was detectable in both proteins.

Proteins containing tryptophan displayed the fluorescence spectrum which is characteristic of this aromatic residue to the exclusion of the tyrosine type of spectrum, even where tyrosine was responsible for most of the absorption at the exciting wavelength. From Table 2, human-serum albumin has relatively the least tryptophan absorption at 280 mμ wavelength. Inspection of the fluorescence spectrum of this protein excited at this wavelength, shown in Fig. 1, reveals a negligible tyrosine contribution at 304 mμ. Comparison of this fluorescence spectrum with that of tryptophan suggests that the quantum yield of the tyrosine fluorescence shown by human-serum albumin cannot be greater than 1·5 %.

Protein–tryptophan fluorescence spectra consisted in all cases of a single band, without the structure sometimes discernible in the absorption spectrum. The excitation spectra were all similar in profile to that of pure tryptophan, with a shift of the band maxima to longer wavelengths ($\lambda_{max.}$ 228 mμ, 282 mμ) compared with tryptophan in water ($\lambda_{max.}$ 218 mμ, 280 mμ).

Fig. 3 (*a*) shows the profiles of the edge of the absorption band of least frequency attributable to tyrosine and tryptophan in proteins. These are obtained from the spectra of proteins in which these aromatic residues predominate and represent average values. Also shown is the variation with wavelength of the fractional absorption of tryptophan in a mixture of tyrosine and tryptophan having equal absorption at 280 mμ wavelength. Fig. 3 (*b*) shows the variation with wavelength of

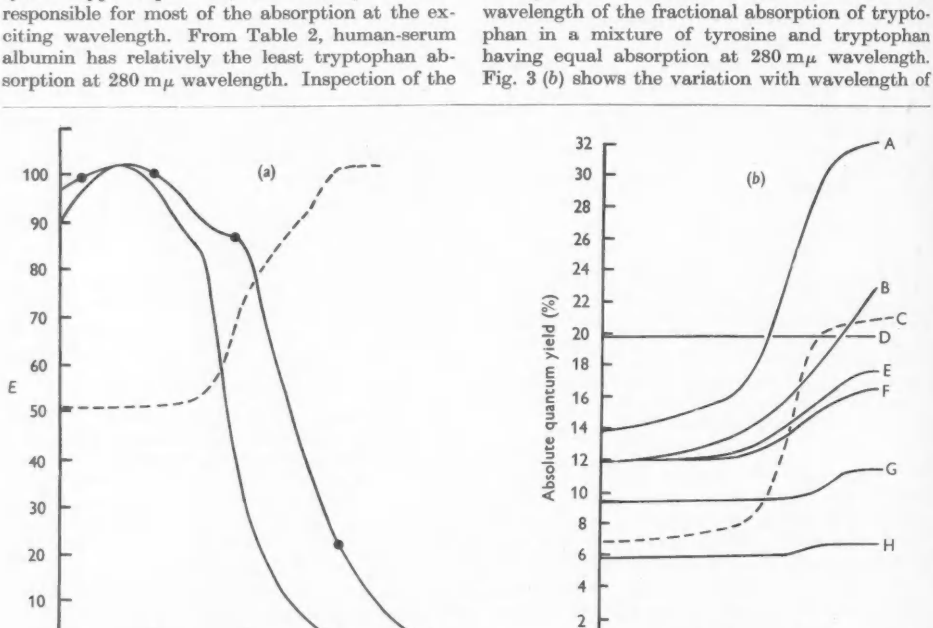

Fig. 3 (*a*). Absorption profiles of tyrosine and tryptophan in proteins between 270 mμ and 310 mμ wavelength. Tyrosine (—); tryptophan (●—●). The fractional absorption by tryptophan in a mixture having equal absorption by both tyrosine and tryptophan at 280 mμ wavelength is shown by the broken line. (*b*) Variation of the fluorescence yield with wavelength of the exciting light in tryptophan and proteins containing tryptophan. A, Bovine-serum albumin; B, pepsin; C, human-serum albumin; D, tryptophan; E, ovalbumin; F, carboxypeptidase; G, chymotrypsin; H, lysozyme.

the absolute fluorescence yield of several tryptophan-containing proteins and of tryptophan in water. These results are considered in more detail in the Discussion section.

Effects of structural changes

Structural changes in proteins produced changes in fluorescence yield and band position. As shown in Table 1, 8M-urea shifted the maxima of all fluorescence spectra of tryptophan-containing proteins to a wavelength of 350 mμ. In some cases it also produced large decreases or increases in fluorescence yield. These changes were sometimes reversed when the urea was removed by dialysis, e.g. chymotrypsin, but the changes were irreversible in other proteins, such as the serum albumins. The specific structural modification involved in the conversion of chymotrypsinogen into the active enzyme was manifested by an increased fluorescence yield and a shifted fluorescence spectrum. 8M-Urea converted both zymogen and enzyme into the same fluorescence parameters, though very slowly with chymotrypsinogen. 0·1M-Calcium chloride reduced the rate of urea-denaturation of chymotrypsin, as measured by the shift of fluorescence spectrum, by a factor of at least 100 (Chervenka, 1959). The trypsinogen–trypsin conversion did not produce large changes in fluorescence, nor did di*iso*propyl phosphorofluoridate-trypsin differ from trypsin.

Increasing the viscosity of the medium by dissolving proteins in propane-1:2-diol almost invariably increased the fluorescence yield, although some proteins were unaffected. Photo-oxidation of the tryptophan content of human-serum albumin in the presence of methylene blue caused the progressive disappearance of all fluorescence; no tyrosine fluorescence was discernible. Treatment of this protein with carboxypeptidase and chymotrypsin produced free tyrosine and tryptophan, which were removed by dialysis. The non-dialysable fraction showed no appreciable tyrosine fluorescence. If the selective removal of tryptophan from albumin was achieved in these experiments, the failure of tyrosine fluorescence to appear indicates a low intrinsic yield for this group in serum albumin.

Quenching of tyrosine

Possible interactions between tyrosine and some of the groups commonly found in proteins were investigated by measuring the fluorescence yield of tyrosine in concentrated aqueous solutions of certain compounds.

Tyrosine was strongly quenched by glycine, glycylglycine, tetraglycine, sodium formate and methyl formate in aqueous solutions and the effects of concentration conformed to the Stern–Volmer equation. The relative concentrations at which the tyrosine yield is reduced to one-half are listed in Table 3. Formamide, urea and ethylamine hydrochloride were ineffective. These results show that strong quenching is produced by the carboxylate group, whereas the charged amino group, amido group and the urea amino groups are without effect. The quenching efficiencies of glycine and related compounds increase with the separation of the charged groups. Surprisingly, the uncharged carboxymethyl group appears almost as effective as the carboxylate ion, although the carbonyl groups in formamide and urea are without effect.

Table 3. *Substances quenching the fluorescence of tyrosine in aqueous solution*

$[q]_{\frac{1}{2}}$ represents that concentration of material at which the fluorescence of μM-tyrosine is halved.

Material	$[q]_{\frac{1}{2}}$ (M)
Tetraglycine	0·110
Sodium formate	0·125
Methyl formate	0·155
Glycylglycine	0·310
Glycine	0·730

The quenching effect of sodium formate was measured with two proteins containing tyrosine. Insulin fluorescence was reduced to half by 0·9M-sodium formate, whereas that of human-serum albumin was unaltered by a 2·0M solution.

DISCUSSION

In the protein molecule each aromatic group can interact in the ground or excited states with adjacent chemical groups of the polypeptide structure, which may be relatively inert paraffin side chains or phenyl groups, or reactive hydrophyllic and charged groups. The displacement of the absorption spectra of tyrosine and tryptophan in proteins compared with aqueous solutions (Beaven & Holiday, 1952) and absorption attributable to tyrosine-carboxylate hydrogen bonds (Tanford, Hauenstein & Rands, 1955) both show that ground-state interactions between these aromatic amino acids and the protein environment occur, which together with possible interactions in the singlet excited state could be expected to reduce the quantum yields shown by these residues in proteins (Teale & Weber, 1959).

The possibilities of energy transfer must also be considered. The relative positions of the absorption and fluorescence spectra should, in favourable circumstances, permit resonance-energy transfer from phenylalanine and tyrosine to tryptophan, and also between adjacent tyrosine groups. Recent fluorescence-polarization measurements with insulin, ribonuclease and zein show clearly that

intertyrosine transfer does indeed occur in these proteins (G. Weber, unpublished work). All the aromatic residues are brought into close proximity by their incorporation into protein molecules, and the average distance between residues is in the range at which efficient transfer is known to take place in other systems (Weber, 1954). For a regular array of aromatic residues, the average spacing, $\bar{D}$ (Å) is given by the expression:

$$\bar{D} = \sqrt[3]{\frac{M}{0{\cdot}602nd}},$$

where M is molecular weight, d is protein density and n is number of aromatic residues in the protein molecule. Values of $\bar{D}$ for the tyrosine groups in the proteins investigated are listed in Table 4. The regular array of groups in the protein to which $\bar{D}$ corresponds is the arrangement least favourable for transfer, so that the more random distribution which undoubtedly exists in proteins should favour the predicted energy transfers. Efficient intertyrosine transfer would permit energy migration throughout those proteins in which this residue predominates. It might be expected that tryptophan fluorescence would be excited by light absorbed by phenylalanine and tyrosine. In fact, phenylalanine fluorescence was not detected in the presence of other aromatic residues, and those proteins containing tryptophan showed the fluorescence of this residue exclusively, irrespective of the relative tyrosine content. The virtual absence of tyrosine fluorescence does not in itself establish complete energy transfer to tryptophan, however; the alternative possibility that tyrosine in proteins may be quenched was suggested by the low fluorescence yields of two trypsin inhibitors and ribonuclease. The investigation of these alternatives was made difficult by the similarity of the absorption spectra of tyrosine and tryptophan. These spectra differ in profile at the absorption minima (approx. 250 mμ) and also at the absorption edge in the wavelength range 280–310 mμ. Measurements at the absorption minimum in proteins were invalidated by the possible transfer from phenylalanine and also by uncertainties of the tyrosine-absorption spectrum in this region.

Table 4. *Average distance* ($\bar{D}$) *between the tyrosine groups in proteins*

Protein	$\bar{D}$ (Å)
Pancreatic trypsin inhibitor	14·73
Zein	15·74
Carboxypeptidase	15·74
Insulin	16·57
Ribonuclease	17·0
Pepsin	17·1
Trypsinogen	18·22
Trypsin	18·22
Human-serum albumin	19·31
Bovine-serum albumin	20·17
Edestin	21·14
Ovalbumin	22·17
Lysozyme	22·51
Ovomucoid	23·33
Chymotrypsinogen	23·33
Chymotrypsin	23·33
Haemoglobin	23·87
Myoglobin	26·54
Bovine γ-globulin	27·05
Fibrinogen	32·30

Less equivocal evidence was furnished by a detailed examination of the variation of tryptophan fluorescence yield over the excitation range 280–310 mμ, where it can be assumed that almost all the absorption is due to tyrosine and tryptophan. In this region the two residues differ in absorption profile, tryptophan absorption extending towards longer wavelengths compared with tyrosine, so that in a mixture of these residues changes in fractional absorption occur, shown in Fig. 3 (*a*). Uniform quantum yields would be observed in this region if efficient energy transfer took place from tyrosine to tryptophan. Experimental results shown in Fig. 3 (*b*) indicate that this is not the case. The large changes in yield observed mean that tyrosine excitation does not contribute appreciably to tryptophan fluorescence. The increases in yield shown in Fig. 3 (*b*) must be regarded as minimal values since it is by no means certain that tryptophan is responsible for all the absorption in the region 295–300 mμ. This is certainly not so when cystine is present in large amounts, as in the serum albumins. By comparison of these yield increases with the fractional absorptions included in Table 2, it is possible to set an upper limit to the amount of transfer present in the proteins investigated. This amount is negligible in all cases except the serum albumins, where transfer to tryptophan from one tyrosine residue at most is indicated. The results of this investigation are in agreement with the two attempts to remove tryptophan preferentially from human-serum albumin. Photo-oxidation progressively reduced the absorption at wavelength 280 mμ and the fluorescence band at 339 mμ without revealing tyrosine fluorescence ($\lambda_{max.}$ 304 mμ). Similarly, the enzymic degradation rapidly liberated dialysable material containing tryptophan, but at no stage was tyrosine fluorescence detectable in the remaining non-dialysable fraction. Both these results suggested that tyrosine residues in albumin are virtually non-fluorescent.

The great similarity between the fluorescence excitation spectra of all these proteins and tryptophan itself rules out any large contribution to protein fluorescence by non-aromatic absorption attributable to cystine, histidine or to the peptide linkage itself, as has been suggested (Konev, 1959).

In Table 1, tryptophan-fluorescence yields, calculated on the assumption of no energy transfer by

dividing the overall yields listed by the tryptophan fractional absorptions listed in Table 2, are included to the right of the overall values in the different media. The wide range of tryptophan-fluorescence yields found in proteins reflects the relative efficiency of quenching processes. For most proteins the yields are reversibly raised by dissolving the protein in propane-1:2-diol, which completes for hydrogen bonds and reduces the rotational freedom of groups on the protein surface. The two serum albumins are unaffected by this treatment, either because the tryptophan groups are in a hydrophobic environment not accessible to the glycol, or because no quenching occurs. The latter possibility is suggested by the high intrinsic yield. In lysozyme, which has a low yield unaltered by glycol, it is unlikely that all eight tryptophan groups cannot be in contact with the medium.

The effect of 8M-urea is more complicated, but generally proteins unaffected by glycol are quenched by 8M-urea, whereas proteins whose yield is increased by glycol are increased by urea, so that the intrinsic tryptophan yields (q_1) become more nearly uniform for all the proteins investigated. With the exception of γ-globulin, lysozyme, haemoglobin and carboxypeptidase, the tryptophan yield in 8M-urea was in all cases $23 \pm 3\%$, and the fluorescence spectra all showed the same band maximum at 350 mμ.

These changes are understandable if urea disrupts the unique protein configuration which provides the specific quenching environment of each tryptophan residue and thus produces a more uniform environment for all the tryptophan groups, and hence a more uniform yield. This effect is clearly seen in the urea denaturation of chymotrypsinogen and chymotrypsin. The fluorescence yields and spectra are originally different but become almost identical after treatment with urea. The fluorescence changes which accompany the activation of chymotrypsinogen are due to a decrease in the quenching of one or more of the tryptophan groups. Presumably, the same structural changes are responsible for the changes in the absorption which are attributable to tyrosine (Chervenka, 1959).

The mechanism responsible for the quenching of tyrosine fluorescence in proteins is suggested by a comparison of insulin with ribonuclease. It is well established that half of the tyrosine phenolic groups in ribonuclease are involved in hydrogen bonds to carboxylate groups, whereas the same groups in insulin appear to be free (Tanford & Epstein, 1954, Tanford *et al.* 1955). The low yield given by ribonuclease (1·7%) compared with insulin (3·7%) is explained if tyrosine is quenched by the interaction with carboxylate. That this is indeed the case is shown by the quenching action of the formate anion and, to a lesser extent, glycine and glycine peptides. Other groups found in proteins, the amide and charged amino groups, seem to be ineffective. The tyrosine-carboxylate hydrogen bond increases the pK of the ionization of the phenolic group (Laskowski & Scheraga, 1954), and the pK of this group in proteins, measured spectrophotometrically, is a measure of the phenolic hydrogen bond. A correlation exists between tyrosine fluorescence and the pK values found in proteins. Insulin shows appreciable fluorescence and a tyrosine pK of 9·7, which is similar to that of free tyrosine. Ribonuclease with roughly half the yield of fluorescence of insulin has half of its tyrosine groups with pK 10·2 and the remainder with pK greater than 11·5. Ovalbumin, in which all the tyrosine groups have a pK greater than 11·5, shows no tyrosine fluorescence. The two serum albumins also show high pK values (Tanford & Roberts, 1952). It must be kept in mind that absence of tyrosine fluorescence does not imply the involvement of all the phenolic groups because energy transfer to quenched tyrosine can decrease the yield of those groups still capable of fluorescence, but measurements indicate that in most of the proteins investigated a large fraction of the phenolic groups is involved in quenching interactions, probably hydrogen bonds to neighbouring carboxylate groups.

Many observations support the view that these phenolic linkages share with cystine the maintenance of the protein tertiary structure. The phenolic dissociation is reversible in the serum albumins and lysozyme, which have relatively high cystine contents (Tanford & Wagner, 1954), but is irreversible in ovalbumin, which has only one cystine bridge (Crammer & Neuberger, 1943) and which is also irreversibly denatured when all the carboxylate groups are titrated at pH 2·0 (Gibbs, Bier & Nord, 1952). Protein-denaturation is frequently accompanied by the freeing of phenolic groups bound in the native protein. For example, heat-denaturation of ribonuclease equalizes the pK values of all six tyrosine groups to the lower value (pH 10·2) shown by only three tyrosine groups in the native protein (Tramer & Shugar, 1959). This agrees with the observation that the fluorescence yield of ribonuclease is doubled by heat-denaturation.

SUMMARY

1. The fluorescence spectra, fluorescence-excitation spectra and absolute quantum yields of 21 globular proteins have been measured in neutral solutions.

2. Phenylalanine fluorescence was not observed in the presence of other aromatic groups, and

tyrosine fluorescence was detected in low yield with band maximum at 304 mμ only in the absence of tryptophan.

3. In proteins containing tryptophan the fluorescence spectrum of this residue alone is observed, irrespective of the wavelength of the exciting light, even in those cases where tyrosine predominates in absorption. The band maxima vary in wavelength between 328 and 342 mμ, and the overall quantum yields 3·8–15·2% in water.

4. Detailed examination of the change in tryptophan quantum yield in the excitation region between 280 and 310 mμ reveals that energy transfer from tyrosine to tryptophan does not take place to any appreciable extent. The similarity of all protein-excitation spectra with that of either tyrosine or tryptophan shows that light absorbed by cystine or the peptide bond does not contribute to protein fluorescence.

5. Modification of the protein structure by 8M-urea produces large and in some cases reversible changes in tryptophan fluorescence, leading to similar yields and fluorescence spectra in most of the proteins examined. The slight structural change involved in the activation of chymotrypsinogen increases the quantum yield by 32%. Propane-1:2-diol increased the yield of all the proteins except the serum albumins and lysozyme.

6. The absence of tyrosine fluorescence in many proteins is explained by the quenching action of the tyrosyl-carboxylate hydrogen bond, and is correlated with the pK values for the phenolic ionization. The role of this linkage in the maintenance of the protein tertiary structure is briefly discussed.

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The Biochemistry of Aromatic Amines

7. THE ENZYMIC HYDROLYSIS OF AMINONAPHTHYL GLUCOSIDURONIC ACIDS*

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As 2-naphthylamine induces cancer of the bladder only, in men exposed to the amine, the actual carcinogen is probably a metabolite of the amine (Boyland, 1958). When tested by implantation in the mouse bladder 2-naphthylamine was much less carcinogenic than one of its metabolites, 2-amino-1-naphthol (Bonser, Clayson, Jull & Pyrah, 1952; Bonser, Clayson & Jull, 1958). However, the 2-amino-1-naphthol has been detected in fresh urine only in conjugated forms (Boyland, 1958) and Boyland (1956) has suggested that it is necessary for the conjugates to be hydrolysed before carcinogenesis occurs. 2-Amino-1-naphthyl sulphate is not hydrolysed by urinary sulphatase (Boyland, Manson, Sims & Williams, 1956) and is not carcinogenic in the mouse bladder (Bonser, Bradshaw, Clayson & Jull, 1956). The corresponding glucosiduronic acid, however, can be hydrolysed by mammalian β-glucuronidase preparations (Boyland & Manson, 1957) and is carcinogenic in the

* Part 6: Boyland & Williams (1959).

mouse bladder (Allen, Boyland, Dukes, Horning & Watson, 1957). Other ways by which 2-amino-1-naphthol might be released in the bladder include the hydrolysis of bis-(2-amino-1-naphthyl) phosphate, which Troll, Belman & Nelson (1959) have detected as a metabolite of 2-naphthylamine in dog urine, and the removal of the sulphate group from 2-amino-1-naphthyl sulphate by means other than by aryl sulphatase.

Since β-glucuronidase may play a part in the induction of bladder cancer after exposure of subjects to 2-naphthylamine, the properties of human urinary β-glucuronidase and its action upon some metabolites of 2-naphthylamine have been investigated.

MATERIALS AND METHODS

Glucuronidase substrates. Phenolphthalein mono-β-glucosiduronic acid (Sigma Chemical Co.) and 8-hydroxyquinoline glucosiduronic acid (California Foundation for Biochemical Research) were commercial samples. 2-Acetamido-1-naphthyl glucosiduronic acid, 2-acetamido-6-naphthyl glucosiduronic acid and 2-amino-1-naphthyl glucosiduronic acid were prepared according to Boyland & Manson (1957). 1-Naphthyl glucosiduronic acid was biosynthesized as described by Berenbom & Young (1951).

Other substrates. 2-Amino-1-naphthyl sulphate was prepared by the persulphate oxidation method of Boyland, Manson & Sims (1953), and 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) according to Roy (1953). 2-Amino-1-naphthyl phosphate was prepared by a perphosphoric acid oxidation of 2-naphthylamine (Boyland & Manson, 1957).

Inhibitors. Ammonium glucosaccharo-1→4-lactone, the 6-methyl and 6-ethyl esters and free glucosaccharo-3→6-lactone were commercial samples from Kemball, Bishop and Co., London.

Determination of enzyme activity

Glucuronidase. The activity of this enzyme is expressed in units: one unit is that which liberates 1 μg. of phenolphthalein in 1 hr. at 37° from phenolphthalein glucosiduronic acid (0·33 mM, pH 4·5), the liberated phenolphthalein being determined colorimetrically at pH 10·5 (Boyland, Wallace & Williams, 1957). 8-Hydroxyquinoline, 1-naphthol, 2-acetamido-1-naphthol and 2-acetamido-6-naphthol were estimated with the Folin–Ciocalteu reagent (Bray & Thorpe, 1954), but 2-amino-1-naphthol could not be determined by this method because it is so easily oxidized. The hydrolysis of 2-amino-1-naphthyl glucosiduronic acid was measured with the Aminco–Bowman spectrophotofluorometer (see below).

Sulphatase. Nitrocatechol liberated from the corresponding sulphate was estimated by the spectrophotometric method described by Dodgson, Melville, Spencer & Williams (1954). Attempts were made to determine the hydrolysis of 2-amino-1-naphthyl sulphate by the benzidine method of Dodgson & Spencer (1953) and by the spectrophotofluorimeter.

Estimations with the Aminco–Bowman spectrophotofluorimeter

The solution to be examined is placed in a quartz cell and a monochromator is used to select a suitable activating wavelength from a xenon arc lamp. Fluorescent light emitted by the sample at right angles to the activating beam is passed through another monochromator on to a photoelectric cell which then excites a galvanometer through a photomultiplier. The instrument permits the detection and estimation of small amounts of fluorescent materials but certain difficulties arise when it is used for the estimation of enzymic activity as in the present case. The most serious is the effect of protein upon the fluorescence. The slight cloudiness of the enzyme solution causes serious errors due to 'scatter' peaks appearing in the fluorescent spectrum. Further, the absorption of ultraviolet light by protein results in a decrease of the apparent fluorescence of the material being examined. These difficulties were overcome by precipitating the protein in the incubation by the addition of 4 vol. of ethanol. The supernatant gave very little scatter and the absorption effect of protein was almost eliminated. The instrument employs a single beam and standard solutions were used to set the reading of the spectrophotofluorimeter during each experiment. Since the unused substrate is being estimated standards are provided by the controls, where ethanol is added immediately after the mixing of enzyme and substrate.

All the 2-amino-1-naphthyl derivatives examined had similar activation and fluorescent spectra, with maximal activation at 325–330 mμ and a fluorescent peak at 410 mμ. The fluorescence of free 2-amino-1-naphthol itself can be seen if precautions are taken to prevent its oxidation. Addition of 4 vol. of ethanol to a solution of mM-2-amino-1-naphthol results in immediate destruction of the free phenol, but the conjugates are stable for at least 1 hr. For the enzyme experiments 0·25 ml. of enzyme solution in buffer was incubated with 0·25 ml. of a substrate solution, and the reaction stopped at the appropriate time by the addition of 2 ml. of ethanol. Appropriate controls were set up in which the enzyme and substrate were mixed just before the addition of the ethanol. The spectrophotofluorimeter was set at approximately 325 mμ activation wavelength and 410 mμ on the fluorescence monochromator and, with one of the controls as the standard fluorescent solution, the wavelength disks were adjusted to give the maximum reading on the photomultiplier galvanometer. The sensitivity of the instrument was then adjusted to give a fixed reading. These adjustments are necessary to correct for fluctuations in the instrument.

In order to check that the decrease in fluorescence was related to the hydrolysis of 2-amino-1-naphthyl derivatives, the hydrolysis of 2-amino-1-naphthyl phosphate by acid phosphatase was followed by this method and by estimation of the phosphate liberated (Fiske & Subbarow, 1925). The two methods were found to be in good agreement.

Anaerobic experiments

These were carried out in Thunberg tubes in an atmosphere of nitrogen. After incubation, 0·5 ml. of the medium was pipetted into 2 ml. of ethanol and the fluorescence of the supernatant determined.

Determination of kinetic constants

These were determined where applicable by the Lineweaver & Burk (1934) method. In the experiments in which the spectrophotofluorimeter was used the decrease in concentration of the substrate was measured and a considerable proportion had to be destroyed before accurate estimations of the hydrolysis could be obtained. In this case the Lineweaver–Burk plot could not be used because it makes the assumption that the concentration of the substrate is substantially constant throughout the experiment. The integrated Michaelis equation of Dixon & Webb (1958) was therefore used.

Inhibition constant of glucosaccharo-1→4-lactone. The K_i values for the lactone were determined by the method of Dixon (1953). At first, when low substrate concentrations were used, non-linear plots were obtained. It was later discovered that this was due to the extremely high affinity of this inhibitor for the enzyme, but this difficulty could be overcome by the use of high substrate concentrations for reasons which will be given later.

Concentration of the urinary enzymes

The β-glucuronidase activity of normal human urine is low (approx. 0·5–2 units/ml.) and the urine frequently contains endogenous inhibitors. Concentration of the enzyme by precipitation of the urine at pH 5·6 with 30% (v/v) of acetone was unsatisfactory as the proportion of the total activity precipitated varied from sample to sample.

The ultrafiltration method of Grant & Everall (1957) using 20 ft. of ⅜ in. flat Visking tubing was more consistent. Cooled, fresh, human male urine (2 l., pooled samples) could be concentrated to 20–30 ml. overnight in the cold. The concentrates were dialysed overnight against distilled water in the cold and, after centrifuging to remove the deposit, preparations containing apparently more than 100% of the original activity of the urine were consistently obtained. Such concentrates had linear dilution–activity relationships showing that they were free from dissociable inhibitors or activators, and were used, unless otherwise stated, in the enzyme experiments described.

Preparation of a concentrate of β-glucuronidase from human kidney

The kidney (205 g., total activity 420 000 units) from a patient with hydronephrosis, which had been removed at operation by Mr D. M. Wallace, was suspended in 300 ml. of ice-cold acetone in a Waring-type blendor. The suspended matter was filtered off and washed with acetone on the filter. The acetone-insoluble material gave 26 g. (total activity 406 000 units) of a buff powder on drying. A 10% (w/v) suspension was made of this powder in water, kept for 15 min. and centrifuged at 600 *g* for 15 min. The supernatant (12 vol.) was diluted with 3 vol. of ice-cold acetone and the precipitate removed by centrifuging after 15 min. A further 5 vol. of acetone was added to bring the acetone concentration to 40% (v/v). This second precipitate was spun down after 15 min. and washed with further acetone. The dried powder was used as a source of β-glucuronidase for comparison with that obtained from urine. The activity was 157 000 units/g., the total activity being approx. 55% of that in the acetone-dried kidney.

Paper electrophoresis

The urine concentrate was run for 18 hr. on a vertically held Whatman no. 100 paper (36 cm. × 5 cm.) at 110 v in the presence of 0·05 M-barbiturate buffer, pH 8·6, 0·1 M-sodium acetate, pH 4·5, or 0·1 M-sodium phosphate, pH 7. The papers were dried at room temperature and the protein was located by staining with bromophenol blue (see Flyn & de Mayo, 1951). Cellulose acetate electrophoresis strips (Oxoid) were also used at pH 8·6. Enzyme activity was determined by cutting the paper into 1 cm. strips and then incubating these strips with 0·33 mM-phenolphthalein glucosiduronic acid in 0·1 M-acetate buffer, pH 4·5, for 18 hr. at 37°.

Cellulose-column chromatography

Carboxymethylcellulose suitable for columns was prepared according to Peterson & Sober (1956). A 10 cm. × 1·75 cm. diameter column, made up in 0·1 M-sodium phosphate buffer, pH 6, was used. The column was loaded with the urine concentrate and eluted with 0·02 M-phosphate buffer, pH 7·5. After overnight dialysis against distilled water, the fractions were incubated with 0·33 mM-phenolphthalein glucosiduronic acid in 0·1 M-acetate buffer, pH 4·5, for 18 hr. at 37° to determine the enzyme-elution band. The protein content of the fractions was estimated by the ultraviolet-absorption method of Warburg & Christian (1941).

RESULTS

Properties of the urinary β-glucuronidase

When the β-glucuronidase activity of whole urine and the enzyme concentrate were measured over 18 hr. at 37·5°, with phenolphthalein mono-β-glucosiduronic acid (4 mM) as substrate, the optimum pH in 0·1 M-acetate buffer was 4·5 for whole urine and 5·0 for the enzyme concentrate. The optimum pH for hydrolysis of 2-amino-1-naphthyl glucosiduronic acid (0·5 mM) by the concentrate under the same conditions was 4·6 (Figs. 1, 2).

Maximum activity of the concentrate occurred at a substrate concentration of 4 mM-phenolphthalein glucosiduronic acid in 0·1 M-acetate at pH 4·5 (Fig. 3). At this optimum substrate concentration the effects of enzyme concentration and time of incubation on the amount of phenolphthalein liberated were determined at pH 4·5. The relationship was linear up to concentrations of approx. 100 units/ml. (1 hr. incubation) and for incubations of up to 20 hr. duration with enzyme activity between 1 and 4 units/ml. Fig. 3 also shows that the hydrolysis of 2-amino-1-naphthyl glucosiduronic acid is directly proportional to the substrate concentration. The reaction is first-order with respect to the substrate concentration, showing that $s \ll K_s$ (equilibrium constant). The substrate concentration (s) is limited by the low solubility of the 2-amino-1-naphthyl glucosiduronic acid.

Talalay, Fishman & Huggins, (1946) found a pH optimum of 4·5 for the tissues of the mouse and rat with phenolphthalein glucosiduronic acid as substrate, but Mills & Paul (1949) reported the presence of two peaks at pH 4·4 and 5·2 with ox-spleen β-glucuronidase.

Enzyme multiplicity. No indication of the presence of more than one β-glucuronidase was obtained from the pH curve (Fig. 1) or by use of paper electrophoresis or carboxymethylcellulose columns (Fig. 4). Further, the enzyme obtained from human kidney was very similar to the urinary β-glucuronidase in its behaviour towards several substrates (Table 1). This would be unlikely if there were more than one enzyme present in either source.

Activation energy. β-Glucuronidase activity at pH 4·5 in 0·1 M-acetate with 4 mM-phenolphthalein glucosiduronic acid as substrate was measured over the range 25–41°. By using the Arrhenius (1889) equation (Fig. 5) the activation energy was found to be 13 600 cal./mole.

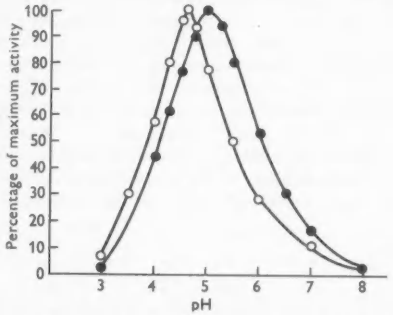

Fig. 1. pH–Activity curves for the β-glucuronidase of a human urine concentrate in the presence of 0·1 M-acetate buffer. ●, Phenolphthalein mono-β-glucosiduronic acid (4 mM); ○, 2-amino-1-naphthyl glucosiduronic acid (0·5 mM). Incubation was for 18 hr. at 37·5°. An enzyme concentration that liberated approximately 1·5 μg. of aglycone/ml./hr. at pH 5 was used with phenolphthalein glucosiduronic acid and a concentration that liberated 20–25 μg./ml./hr. at pH 4·6 with 2-amino-1-naphthyl glucosiduronic acid.

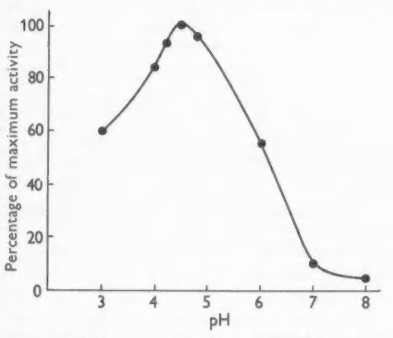

Fig. 2. pH–Activity curve for the β-glucosiduronidase of whole urine in the presence of 0·1 M-acetate buffer. The substrate was 4 mM-phenolphthalein mono-β-glucosiduronic acid and incubation was for 18 hr. at 37·5°. Activity of the urine was 1–1·5 units/ml.

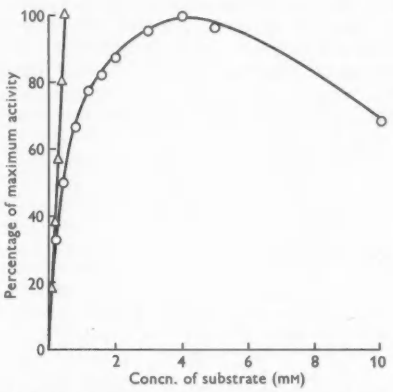

Fig. 3. Substrate concentration–activity curve for the β-glucuronidase of human-urine concentrate in the presence of 0·1 M-acetate buffer, pH 4·5. Substrates: ○, phenolphthalein mono-β-glucosiduronic acid; △, 2-amino-1-naphthyl glucosiduronic acid; incubation was for 18 hr. at 37·5°. The enzyme concentrations used were similar to those in Fig. 1. (For comparison of the relative rates of hydrolysis of the two substrates see text.)

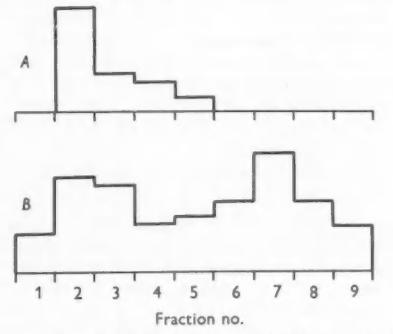

Fig. 4. Elution of β-glucuronidase activity of urine concentrate from a carboxymethylcellulose column with 0·02 M-phosphate buffer, pH 7·5. Each fraction was 3·9 ml. Fraction 2 contained 52 units of enzyme and 9·2 mg. of protein. *A*, β-Glucuronidase activity; *B*, protein concentration.

Kinetic constants. Michaelis constants and $V_{max.}$ for a number of glucosiduronic acids were determined at pH 4·5 in 0·1 M-acetate buffer after incubation for 18 hr. at 37·5°. The results are shown in Table 1.

No hypothesis on the nature of the enzyme action can be advanced since the quantitative effect of the aromatic radicals upon the ether link of the glucosiduronic acid is difficult to estimate with the substrates used. It is, however, of interest that with the exception of 1-naphthyl glucosiduronic acid the K_m values are more influenced by the nature of the substrate than are the $V_{max.}$ values. The aglycone has more effect on the free substrate than on the enzyme–substrate complex.

The rate of enzymic hydrolysis of 2-amino-1-naphthyl glucosiduronic acid was measured by determining the destruction of the substrate, and the integrated Michaelis equation of Dixon & Webb (1958) was used in an attempt to determine K_m and $V_{max.}$ for this substrate. Here the assumption was made that the products of the reaction do not have a significant effect upon the reaction rate, but this might not be justifiable, since a considerable proportion (30–50 %) of the substrate was destroyed. However, one of the products of the reaction, 2-amino-1-naphthol, is easily oxidized so that it is probably destroyed during an aerobic incubation for 18 hr. Experiments were also carried out under anaerobic conditions. Under both conditions slight positive slopes were obtained (Fig. 6), whereas a negative slope would be expected for the normal case. Dixon & Webb (1958) have, however, shown that in regions where the substrate concentration is not much greater than the Michaelis constant a line with zero slope is obtained. The concentration of 2-amino-1-naphthol glucosiduronic acid is restricted to low values by its limited solubility. The experiments appear therefore to have been carried out in the region where zero slopes might be obtained, and the values of K_m or $V_{max.}$ for 2-amino-1-naphthyl glucosiduronic acid cannot be determined by this method alone. The slight increase in the reaction rate under aerobic conditions, as compared to the anaerobic experiments, may be a result of the removal of 2-amino-1-

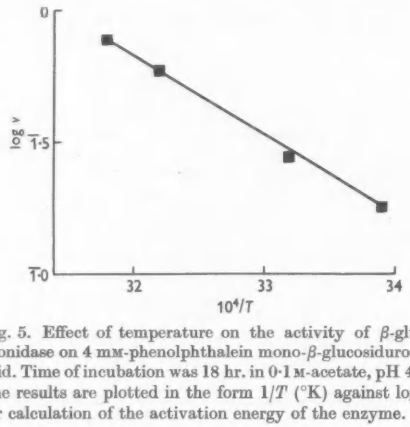

Fig. 5. Effect of temperature on the activity of β-glucuronidase on 4 mM-phenolphthalein mono-β-glucosiduronic acid. Time of incubation was 18 hr. in 0·1 M-acetate, pH 4·5. The results are plotted in the form $1/T$ (°K) against log v for calculation of the activation energy of the enzyme.

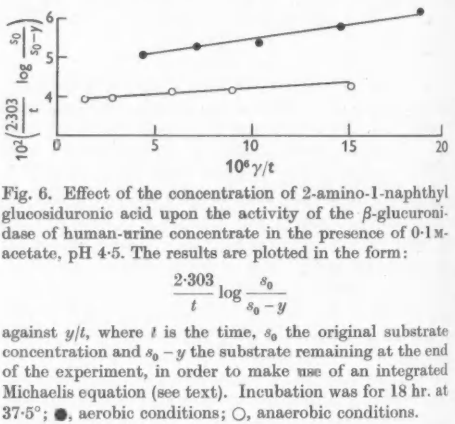

Fig. 6. Effect of the concentration of 2-amino-1-naphthyl glucosiduronic acid upon the activity of the β-glucuronidase of human-urine concentrate in the presence of 0·1 M-acetate, pH 4·5. The results are plotted in the form:

$$\frac{2{\cdot}303}{t}\log\frac{s_0}{s_0-y}$$

against y/t, where t is the time, s_0 the original substrate concentration and $s_0 - y$ the substrate remaining at the end of the experiment, in order to make use of an integrated Michaelis equation (see text). Incubation was for 18 hr. at 37·5°; ●, aerobic conditions; ○, anaerobic conditions.

Table 1. *Hydrolysis of glucosiduronic acids by the β-glucuronidase from human male urine and human kidney*

The $V_{max.}$ values are quoted relative to that obtained with the urine enzyme and phenolphthalein glucosiduronic acid. Incubates contained 0·1 M-acetate buffer, pH 4·5; the time of incubation was 18 hr. at 37·5°. Because of the limited solubility of the substrate and its low affinity for the glucuronidase, the K_m and $V_{max.}$ values could not be determined for 2-amino-1-naphthyl glucosiduronic acid.

Substrate	$10^4 K_m$ (M)		$V_{max.}$	
	Urine	Kidney	Urine	Kidney
Phenolphthalein glucosiduronic acid	3·3	3·6	100	105
8-Hydroxyquinoline glucosiduronic acid	8·3	7·7	114	110
2-Acetamido-6-naphthyl glucosiduronic acid	13·8	15·4	85	91
2-Acetamido-1-naphthyl glucosiduronic acid	50	50	99	102
1-Naphthyl glucosiduronic acid	29·4	—	32	—

naphthol. Some quenching of the fluorescence of the substrate by the products of the reaction could result in the slight positive slopes. It was not possible to determine K_i (which might approximate to K_m and thus with the $V_{max.}/K_m$ value already obtained give $V_{max.}$) for 2-amino-1-naphthyl glucosiduronic acid when acting as a competitive inhibitor for phenolphthalein glucosiduronic acid. The low solubility and affinity of the 2-amino-1-naphthyl glucosiduronic acid did not allow sufficient inhibition to be obtained for estimation of this kinetic constant. This result supports the reasons given for the almost zero slopes obtained with the integrated Michaelis equation.

Despite the low affinity of the β-glucuronidase for 2-amino-1-naphthyl glucosiduronic acid, this substrate has an appreciable rate of hydrolysis. In 0·1 M-acetate, pH 4·5, the rate of substrate destruction, on a molar basis, of phenolphthalein glucosiduronic acid (0·33 mM) and 2-amino-1-naphthyl glucosiduronic acid (0·33 mM) was 1:1·26 for 18 hr. incubations, under aerobic conditions. Under anaerobic conditions the ratio was 1:0·93.

Inhibition of the β-glucuronidase by glucosaccharo-1 → 4-lactone and the 6-methyl and 6-ethyl esters of the lactone

Glucosaccharo-1 → 4-lactone is a powerful competitive inhibitor of mouse-liver β-glucuronidase (Levvy, 1952) and its action upon the human urinary enzyme was examined. Initial attempts to determine the inhibition constant by the method of Dixon (1953) gave non-linear plots. If phenolphthalein was incubated with the urine concentrate at pH 4·5 complete recoveries as shown colorimetrically were obtained. Combination of substrate and inhibitor (see Friedenwald & Maengwyn-Davies, 1954) did not seem a probable explanation because the concentration of substrate was over 100 times as great as that of the inhibitor and each inhibitor molecule would have to combine with several molecules of substrate to give the degree of inhibition obtained. Thus inhibition was almost certainly due to enzyme–inhibitor interaction.

The Michaelis–Menten equation, upon which the normal methods of determining equilibrium constants are based, makes the assumption that the concentration of the substrate or inhibitor combining with the enzyme is very much greater than the concentration of the enzyme-active sites. Glucosaccharo-1 → 4-lactone has a very high affinity for β-glucuronidase and thus could be acting in a pseudo-irreversible manner under the conditions (substrate concentration 0·25, 0·5 and 1 mM; inhibitor concentrations 0·2–2 μM) used in the initial attempts to determine K_i.

Extensions of the original Michaelis–Menten treatment to include such cases have been put forward by Strauss & Goldstein (1943), Goldstein (1944) and Ackermann & Potter (1949). The last-named authors describe a simple graphical method for determining whether the Michaelis–Menten equation is applicable to a particular case. Plotting of the rate of reaction against the enzyme concentration (substrate 75 μM, inhibitor 1 μM) in the presence and absence of inhibitor gave the pseudo-irreversible inhibitor plot of Ackermann and Potter, i.e. the inhibitor line cuts the X axis to the right of the origin.

For reasons of economy low concentrations of phenolphthalein glucosiduronic acid well below the optimum had been employed. Substrate concentrations nearer the optimum, however, enable smaller enzyme concentrations to liberate sufficient phenolphthalein for accurate estimation. Higher concentrations of substrate also allow the use of higher inhibitor concentrations. Thus the relative enzyme concentration is decreased and the inhibitor concentration increased so that the system moves away from the pseudo-irreversible zone. An Ackermann–Potter graph at high substrate concentrations (substrate 3·75 mM, inhibitor 10 μM) now gave the plot for reversible inhibition, i.e. both in the absence and presence of the inhibitor, straight lines which pass through the origin were obtained.

When substrate concentrations of 3·75 and 1·88 mM and inhibitor concentrations (i) of 10–50 μM were used linear plots of $1/v$ against i were obtained, showing glucosaccharo-1 → 4-lactone to be a competitive inhibitor (Dixon, 1953) with a K_i of 1·6 μM for human urinary β-glucuronidase.

From these results it appears that the original system used for determining K_i was in the region where the assumption made in the Michaelis–Menten equation was becoming unjustifiable; for by altering the concentrations of enzyme, substrate and inhibitor (which for practical reasons are dependent upon each other) it was possible to push the systems into regions where (*a*) the Michaelis–Menten equation was applicable, (*b*) where the enzyme-inhibitor reaction was pseudo-irreversible.

Glucosaccharo-1 → 4-lactone has been administered to patients from whom bladder tumours have been removed in order to reduce the urinary β-glucuronidase activity (Boyland *et al.* 1957). The effect of the lactone was measured by determining the fall in β-glucuronidase activity of the urine at the optimum pH 4·5. Now the pH of human urine (Dr H. Davies, personal communication) can vary from 5 to above 7, the mode being about 5·8. Figs. 7 and 8 show the effect of pH upon the inhibition by saccharolactone of the β-glucuronidase in a urine concentrate. The inhibition falls away from that at the optimum pH. However, it is still appreciable at pH 5·8 and the saccharolactone exerts its greatest action where it is most required,

i.e. when the enzyme is most active (Boyland, Davies & Williams, 1959). The measurement of the activity of the urine at pH 4·5 can, however, give a misleading impression of the actual activity in the bladder.

The esters of glucosaccharo-1 → 4-lactone were much less potent inhibitors of β-glucuronidase. The 6-methyl ester was shown by the method of Dixon (1953) to be non-competitive with K_i 1 mM. The 6-ethyl ester was not an inhibitor.

In all the inhibitor experiments the lactones were made up just before use. Incubation was for 1 hr. in 0·2 M-acetate buffer, the reaction being stopped and the phenolphthalein colour developed by the addition of 10 % (anhydrous w/v) sodium carbonate solution.

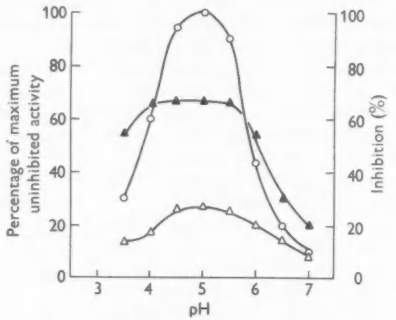

Fig. 7. Effect of pH upon the inhibition of the β-glucuronidase of human-urine concentrate by glucosaccharo-1→4-lactone. Incubation was for 1 hr. at 37·5°, and the buffer was 0·2 M-acetate. Substrate 330 μM-phenolphthalein glucosiduronic acid, inhibitor concentration 10 μM. ○, pH–activity curve in the absence of inhibitor; △, pH–activity curve in the presence of inhibitor; these results are expressed as a percentage of the maximum uninhibited activity; ▲, inhibition expressed as a percentage of the activity at the same pH in the absence of inhibitor.

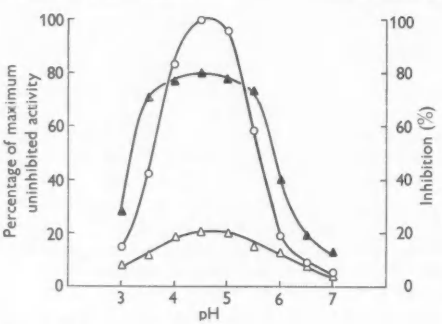

Fig. 8. As Fig. 7 but with 330 μM-2-amino-1-naphthol glucosiduronic acid used as the substrate. In calculating the activity, account has been taken of the destruction of a large proportion of the total substrate concentration (see text). Incubation was for 18 hr.

Sulphatase

Boyland *et al.* (1956) showed that no free 2-amino-1-naphthol could be detected by the Folin–Ciocalteu reagent after incubation of 2-amino-1-naphthyl sulphate with urinary sulphatase and this observation has been confirmed by independent methods. Attempts were made to estimate sulphate liberation by the benzidine method of Dodgson & Spencer (1953), but the sulphate liberated from 0·5 mM-2-amino-1-naphthyl sulphate at pH 5·6 in 0·1 M-acetate buffer was less than 1 % of that liberated from nitrocatechol sulphate under the optimum conditions (0·03 M, pH 5·6, 0·25 M-buffer; Dodgson & Spencer, 1957). The spectrophotofluorimetric estimations also showed that the rate of destruction of 2-amino-1-naphthyl sulphate was less than 1 % of nitrocatechol sulphate under these conditions.

DISCUSSION

After consideration of the carcinogenic properties of metabolic derivative of aromatic amines, Boyland (1959) suggested that 2-amino-1-naphthyl glucosiduronic acid or bis-(2-amino-1-naphthyl)phosphoric ester could be the causes of bladder cancer resulting from exposure to 2-naphthylamine. The actual carcinogen was probably 2-amino-1-naphthol (or an oxidation product) liberated in the bladder by the action of the urinary β-glucuronidase, or other hydrolytic process. Use of a concentrate of human urinary β-glucuronidase has shown that the enzyme will hydrolyse 2-amino-1-naphthyl glucosiduronic acid at about the same rate as the phenolphthalein conjugate. Further evidence was obtained that the 2-amino-1-naphthyl sulphate was inert to the action of urinary sulphatases, this being a possible explanation for its not having a carcinogenic action in the mouse bladder as does the glucosiduronic acid (Allen *et al.* 1957).

The inhibition of human β-glucuronidase at low concentrations of substrate and of glucosaccharo-1 → 4-lactone has been found to be pseudo-irreversible. However, unlike the case of physostigmine and cholinesterase considered by Strauss & Goldstein (1943), a pseudo-irreversible reaction would not occur in the urine of patients with bladder cancer undergoing prophylactic treatment with saccharolactone. β-Glucuronidase is present in low concentration in urine whereas cholinesterase is very highly concentrated at the motor end-plates. Despite its very high affinity therefore it would seem that an inhibitor of increased affinity would be still more effective in suppressing the β-gluc-

uronidase activity of urine. The main disadvantage of saccharolactone is, however, that it is so rapidly metabolized by the body that only a small proportion of the dose is excreted.

Consideration of the effect of pH upon the total activity and the percentage inhibition must be given since both fall sharply away from values at the optimum pH. In the testing of urine for activity its original pH as well as the activity at pH 4·5 should be noted in order that a more exact indication of the activity *in vivo* is obtained. Measurement at the pH of the urine would be difficult because of its low activity.

There has been no evidence of the abnormality in the mammalian enzyme comparable with that reported for the β-glucuronidase of *Cellana tramoseria* (Cox, 1959). The pH optimum of the concentrate remains constant with variation in substrate concentration. There was, however, a slight shift in the optimum pH from 4·5 to 5 on concentration of the enzyme. No evidence for the presence of more than one enzyme was discovered.

Structure of ammonium glucosaccharo-1 → 4-lactone

Although the lactone is such a potent inhibitor of β-glucuronidase it has been assigned a 1:4 ring (Smith, 1944), whereas bornyl glucosiduronic acid (Pryde & Williams, 1933) and 2-naphthyl glucosiduronic acid (Tsou & Seligman, 1952) have been shown, by independent methods, to possess 1:5-pyranoside rings. Marsh & Levvy (1958) have also synthesized aryl β-D-glucopyranosiduronic acids by the same general method (catalytic oxidation of hexosides) as Tsou & Seligman (1952) and show that the hydrolysis of these conjugates by β-glucuronidase is inhibited by saccharolactone. The evidence presented by Levvy (1952) and that in this paper show that only slight changes in the saccharolactone molecule greatly reduce its affinity for the enzyme. This stereochemical specificity may throw some doubt upon the ring structure given by Smith (1944), since this has not been confirmed by independent methods, unlike the structure of the aryl β-D-glucopyranosiduronic acids.

SUMMARY

1. β-Glucuronidase of human urine, prepared by ultrafiltration and dialysis, hydrolyses the *O*-glucosiduronic acid derivatives of phenolphthalein, 8-hydroxyquinoline, 2-acetamido-6-naphthol, 2-acetamido-1-naphthol, 1-naphthol and 2-amino-1-naphthol, although 2-amino-1-naphthyl glucosiduronic acid has a lower affinity than the other substrates.

2. The theoretical maximum velocity of hydrolysis ($V_{max.}$) for 2-amino-1-naphthyl glucosiduronic acid could not be determined; that for 1-naphthyl glucosiduronic acid was a third of that of other substrates.

3. The affinity of glucosaccharo-1 → 4-lactone for β-glucuronidase is 1000 times as great as that of its 6-methyl and 6-ethyl esters. The inhibition of β-glucuronidase by glucosaccharo-1 → 4-lactone is much greater in the region of the optimum pH of the enzyme.

4. The resistance of 2-amino-1-naphthyl sulphate to hydrolysis by aryl sulphatase has been confirmed.

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Biochem. J. (1960) **76**, 396

Cobalamin and the Synthesis of Methionine by Ultrasonic Extracts of *Escherichia coli*

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The synthesis of methionine from homocysteine and serine by *Escherichia coli* has been studied in these laboratories with suspensions of intact organisms (Gibson & Woods, 1960) and with enzymic extracts (Cross & Woods, 1954; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). With whole organisms strong presumptive evidence was obtained that some form of folic acid was required as coenzyme for the C_1-unit transfer and this was confirmed by the studies with enzyme preparations, though tetrahydropteroylglutamic acid and other forms of folic acid tried were not effective sources of the folic acid coenzyme (Cross & Woods, 1954; Szulmajster & Woods, 1955, 1960). Extracts of acetone-dried organisms were, however, activated by tetrahydropteroylglutamic acid provided that the organisms had been harvested from a medium containing cobalamin (Kisliuk & Woods, 1957, 1960).

Using an auxotrophic strain of *E. coli* requiring methionine or cobalamin for growth, Gibson & Woods (1952, 1960) found that washed suspensions of organisms harvested from a medium devoid of cobalamin did not synthesize methionine unless the vitamin was added. The formation of methionine by suspensions of a number of strains of *E. coli* not requiring cobalamin for growth was also increased markedly by the vitamin, the largest effect (over fivefold) being obtained with an auxotrophic strain (PA 15) with a growth requirement for serine or glycine. Most of the work with enzymic extracts quoted above was done with extracts of acetone-dried powders of this strain and no direct effect of cobalamin on the activity of the extracts was found.

In the present work the primary enzyme preparations were made by ultrasonic vibration instead of by drying with acetone. An effect of cobalamin was at once apparent under all conditions with a mutant requiring cobalamin or methionine for growth (and grown with methionine). Cobalamin now also activated methionine synthesis under certain conditions with strain PA 15; it enabled tetrahydropteroylglutamate to serve as a source of the folic acid coenzyme also required in the reaction. Brief accounts of part of this work have already been published (Helleiner & Woods, 1956; Helleiner, Kisliuk & Woods, 1957; Woods, 1958). Towards the end of this work a brief account appeared of a cobalamin-containing enzyme, forming part of the complex of enzymes (in a cobalamin auxotroph of *E. coli*), which catalyses the transfer of C-3 of serine to homocysteine (Hatch, Takeyama & Buchanan, 1959).

There have been several investigations of the formation of methionine from homocysteine by enzymic extracts of chicken, sheep and pig liver with serine or formaldehyde as donor of the C_1 unit (Doctor, Patton & Awapara, 1957; Nakao & Greenberg, 1958; Stevens & Sakami, 1959). No effect of cobalamin was described; tetrahydropteroylglutamic acid was an effective source of folic acid with sheep- and pig-liver preparations and pteroylglutamic acid itself was active with the enzyme from chicken liver.

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MATERIALS AND METHODS

Organisms and enzymic extracts

Organisms. Stock cultures of all organisms were maintained on slopes of tryptic meat–agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. The greater part of the work was done with two auxotrophic strains of *E. coli*. Strain 121/176, which requires cobalamin or methionine for growth, was as described by Davis & Mingioli (1950) and was obtained from Dr B. D. Davis. Strain PA15 requires glycine or serine and was originally obtained from Dr Barbara Wright; it has been used in previous studies of methionine synthesis in these laboratories (Gibson & Woods, 1960; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). Other strains used in a few experiments were 113/3, another cobalamin or methionine auxotroph (Davis & Mingioli, 1950), and two prototrophs, W (the parent strain of 121/176 and 113/3) and 518 (ATCC no. 9723).

Media and growth. The basal medium for bulk growth contained (g./l.): K_2HPO_4, 19·2; KH_2PO_4, 8·8; $(NH_4)_2SO_4$, 2·0; NH_4Cl, 5·0; $MgSO_4,7H_2O$, 0·2; glucose, 4·0; lactic acid, 20·0. After addition of Fe^{2+} ions (final concn. 8 μM) from a solution of $FeSO_4,(NH_4)_2SO_4,6H_2O$ in twice its molar concentration of sodium citrate, the pH was brought to 7 with 10N-NaOH. The medium was supplemented with DL-methionine (0·5 mM) for strains 121/176 and 113/3, and with glycine (10 mM) for strain PA15. After addition of 3% (w/v) of agar and sterilization (121°; 10 min.) the medium was dispensed either in Roux bottles (100 ml.) or enamelled trays (as described by Szulmajster & Woods, 1960) (600 ml.).

In a few experiments cobalamin replaced methionine as growth factor for strain 121/176 or was added to the complete medium for strain PA15; it was added as a sterile solution to the bulk medium.

The inoculum for a Roux bottle was about 1·5 mg. dry wt. of organisms from a tryptic meat–agar slope harvested after incubation for 16 hr. at 37°. Each tray received about 3 mg. dry wt. of organisms from a 16 hr. culture (37°) on minimal medium (Davis & Mingioli, 1950) supplemented with any required growth factor. Both trays and Roux bottles were incubated for 44 hr. at 37°. The organisms were harvested, washed twice in the centrifuge with either water or 0·85% NaCl solution (140 ml./tray; 45 ml./Roux bottle), and finally suspended in either 0·2M-phosphate buffer, pH 7·3, or 0·05M-phosphate buffer, pH 7·8, according to the reaction mixture used for studying methionine synthesis (see below).

Preparation of enzymic extracts. Washed suspensions (10–25 ml.) containing 75–100 mg. dry wt. of organisms/ml. were subjected to ultrasonic vibration (25 kcyc./sec., 4 min., 600 w) in a cooled, stainless-steel, conical flask, to the base of which the magneto-strictor was brazed. The ultrasonic generator was type E7590B of Mullard Ltd., London, W.C. 1. After centrifuging at 0° (25 000 *g*/10 min.) the supernatant fluid was dialysed either against running tap water overnight or against two changes of 2 l. quantities of 0·05M-phosphate buffer, pH 7·8, at 3°. The dialysed preparation was centrifuged to remove precipitated protein and the protein content of the supernatant (which was used as enzyme source) was estimated spectrophotometrically according to Layne (1957).

In some experiments the dialysed extract was treated with Dowex-1 resin; the procedure was that of Kisliuk & Woods (1960), except that the resin (chloride form) was X8, 100–200 mesh.

The enzyme preparations were normally used within 2 days of preparation since that from strain 121/176 progressively lost activity (50% in 3 weeks) when stored at either 2° or −15°; the preparation from strain PA15 was more stable (15% loss in 3 weeks).

Extract of heated organisms. Washed suspensions (170 mg. dry wt./ml.) of the strain under test were heated for 3 min. at 100°, cooled and centrifuged. The supernatant will be referred to as extract of heated *E. coli*; it contained 0·15 μmole of methionine/ml.

Assessment of mass of organisms. This was estimated photometrically as described by Gibson & Woods (1960).

Test for methionine synthesis

Reaction mixtures. The solutions used for testing methionine synthesis by ultrasonic extracts were based on the work of Cross & Woods (1954) and Kisliuk & Woods (1960). Basal reaction mixture *A* was used for most of the work with *E. coli* 121/176; it contained, in 2 ml. of potassium phosphate buffer (0·05M, pH 7·3): DL-serine, 20 μmoles; DL-homocysteine, 20 μmoles; glucose, 20 μmoles; $MgCl_2$, 10 μmoles; adenosine triphosphate (ATP), 4 μmoles; diphosphopyridine nucleotide (DPN), 0·4 μmole. Basal reaction mixture *B* was used mainly with *E. coli* PA15 but also in the later work with strain 121/176. It contained [in 2 ml. of potassium phosphate buffer (0·125M, pH 7·8)]: L-serine, 10 μmoles; DL-homocysteine, 10 μmoles; glucose, 20 μmoles; $MgCl_2$, 10 μmoles; ATP, 10 μmoles; DPN, 1 μmole; pyridoxal phosphate, 0·5 μmole. In experiments with the formaldehyde derivative of tetrahydropteroylglutamic acid as the C_1-unit donor the amount of DL-homocysteine was decreased to 5 μmoles and pyridoxal phosphate was omitted (Kisliuk & Woods, 1960).

When present, extract of heated *E. coli* was added to both reaction mixtures in an amount equivalent to 34 mg. dry wt. of the original organisms. The amount of enzymic extract used as a routine contained 10 mg. of protein.

The complete reaction mixture was incubated at 37° for 4–6 hr. and normally under an atmosphere of H_2, though static incubation in air was sometimes used when tetrahydropteroylglutamic acid was not present. After heating (100°, 3 min.) the reaction mixture was centrifuged to remove precipitated protein and the supernatant fluid used for the assay of methionine.

Estimation of methionine. Microbiological assay with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 was carried out as described by Gibson & Woods (1960) with DL-methionine as standard; the organism responds only to the L-isomer. Additional information on the specificity of the assay is given by Kisliuk & Woods (1960).

Expression of results. The amount of L-methionine formed is given throughout in terms of μm-moles/mg. of protein of the enzyme preparation in the time incubated; results are not given per unit of time since in some cases there was a lag in methionine formation during the first hour of incubation.

A control in which a reaction mixture containing all components was heated at zero time was included in each

experiment; the value obtained (about 4 μm-moles of L-methionine/mg. of protein) was deducted from the experimental and other control values.

Chemicals

Cobalamin and derivatives. The source of cobalamin was Anacobin (British Drug Houses Ltd., Poole, Dorset). It was about 90% pure when assayed spectrophotometrically with the extinction coefficient quoted by Brink *et al.* (1949).

Pseudovitamin B_{12}, factors A, F and III and the 5-methylbenziminazole, 5:6-dichlorobenziminazole and 2:6-diaminopurine analogues of cobalamin were provided by Dr J. E. Ford; factor B was given by Dr E. Lester Smith. The chemical nature and biological properties of these members of the vitamin B_{12} group are summarized by Kon & Pawelkiewicz (1958).

The specimen of 5:6-dimethylbenzimidazolylcobamide coenzyme was given by Dr H. A. Barker. In experiments with this compound precautions were taken at all stages to exclude light.

Irradiated specimens of both cobalamin and 5:6-dimethylbenzimidazole B_{12}-coenzyme were prepared by exposing 0·3 ml. of a 10 μM-solution in a silica cell for 50 min. at 6 ft. from an 80 w white fluorescent light. This was sufficient to cause the changes in absorption spectrum reported by Barker, Weissbach & Smyth (1958).

Folic acid derivatives. Tetrahydropteroylglutamic acid (tetrahydro-PtGA) and its formaldehyde derivative were prepared and standardized by methods quoted by Kisliuk & Woods (1960). The formaldehyde derivative (which will be referred to as tetrahydro-PtGA–CH_2O) is believed to be N^5N^{10}-methylenetetrahydropteroylglutamic acid (Kisliuk, 1957; Blakley, 1959).

Other chemicals. Adenosine triphosphate, diphosphopyridine nucleotide (and its reduced form, $DPNH_2$) and triphosphopyridine nucleotide (TPN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. The DL-homocysteine was from either Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. or Mann Research Laboratories Inc., New York, N.Y., U.S.A.

RESULTS

E. coli 121/176 is an auxotroph requiring either cobalamin or methionine for growth. In order to obtain organisms and enzyme preparations which could be expected to be relatively deficient in cobalamin it was grown with methionine as supplement; concentrations only just optimum for growth were used since higher ones were likely to repress the formation of the enzyme systems under investigation (Wijesundera & Woods, 1960). Strain PA 15 does not require cobalamin for growth, but synthesis of methionine by suspensions of whole organisms is increased several-fold by the vitamin (Gibson & Woods, 1960).

The effect of cobalamin on the synthesis of methionine from homocysteine by ultrasonic extracts of both strains was examined under three sets of conditions with regard to the nature of the

Table 1. *Effect of cobalamin when extract of heated* Escherichia coli *is the source of folic acid coenzyme*

Strain 121/176: reaction mixture *A* plus enzymic extract, extract of heated *E. coli* and cobalamin (14 $\mu\mu$moles). Incubated for 4 hr.

Strain PA 15: reaction mixture *B* plus Dowex-treated enzymic extract and extract of heated *E. coli*. Cobalamin, 70 $\mu\mu$moles, when present. Incubated for 4 hr.

Modifications to reaction mixtures		L-Methionine formed (μm-moles/mg. of protein)	
Omissions	Additions	Strain 121/176	Strain PA 15
None	—	24	76
None	Cobalamin	—	75
Cobalamin	—	6	—

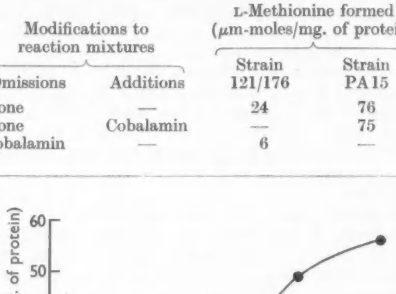

Fig. 1. Effect of cobalamin on the rate of formation of methionine by enzymic extracts of *E. coli* 121/176 when extract of heated organisms is the source of folic acid coenzyme. Reaction mixture *A* plus enzymic extract, extract of treated *E. coli* and cobalamin (when present), 14 $\mu\mu$moles. ●, Complete system; ○, no cobalamin; ◒, no DL-homocysteine.

Table 2. *Effect of the concentration of cobalamin on methionine synthesis by extracts of* Escherichia coli 121/176

Reaction mixture *B* plus enzymic extract and either extract of heated *E. coli* or tetrahydro-PtGA (1 μmole). Incubated for 4 hr.

Cobalamin added ($\mu\mu$moles)	L-Methionine formed (μm-moles/mg. of protein)	
	Extract of heated *E. coli*	Tetrahydro-PtGA
0	6	3
14	18	25
28	25	40
70	40	62
350	44	88

donor of the C_1 unit and the nature of the source of the coenzyme form of folic acid (CoF): (*a*) serine with extract of heated *E. coli*, (*b*) serine with tetrahydropteroylglutamic acid, and (*c*) the formaldehyde derivative of tetrahydro-PtGA (tetrahydro-PtGA–CH_2O) as both donor and CoF source. For reasons discussed by Kisliuk & Woods (1960) it is not possible to use free formaldehyde as a donor of C_1 units when homocysteine is present.

The activity of the enzymic extracts of both strains varied overall about threefold from one preparation to another. This may be due to a number of enzymes with varying stability being required for the overall reaction: the work of Emerson (1950) with mutants of *Neurospora* suggests that the reaction may be complex.

Effect of cobalamin

Serine with extract of heated E. coli. Under these conditions methionine was synthesized by extracts of strain PA 15 in the absence of cobalamin and its addition did not improve the yield. On the other hand, synthesis by strain 121/176 was increased about fourfold by cobalamin (Table 1); after a slight initial lag methionine formation was proportional to time for 4 to 6 hr. and was negligible in the absence of homocysteine (Fig. 1). The effect of cobalamin concentration (of which an example is given in Table 2) was variable in the sense that the higher concentrations did not always give further increases in methionine synthesis; with some enzyme preparations, however, the increases were considerably greater than those shown in Table 2.

Serine with tetrahydropteroylglutamic acid. Cobalamin was required for methionine synthesis not only by the cobalamin-deficient enzymic extracts of strain 121/176 but also by the extracts of strain PA 15 (Table 3 and Fig. 2). The rate of methionine synthesis with strain PA 15 (Fig. 2) shows an initial lag of about 1 hr. before becoming almost linear up to 4 hr.; the lag period was usually decreased if a much larger quantity of cobalamin (about 1 μm-mole) was used, although the maximum rate of methionine formation was not necessarily increased.

The effect of concentration of cobalamin with tetrahydro-PtGA present is shown in Table 2 for

Table 3. *Requirement for cobalamin when tetrahydropteroylglutamic acid is the source of folic acid coenzyme*

Reaction mixture *B* plus Dowex-treated enzymic extracts and either extract of heated *E. coli* or tetrahydro-PtGA (1 μmole). Incubated for 4 hr. Cobalamin: +, present (70 $\mu\mu$moles); −, absent.

Source of CoF	Cobalamin	L-Methionine formed (μm-moles/mg. of protein) Strain 121/176	Strain PA 15
Extract of heated *E. coli*	+	40	55
Tetrahydro-PtGA	−	3	5
Tetrahydro-PtGA	+	62	80

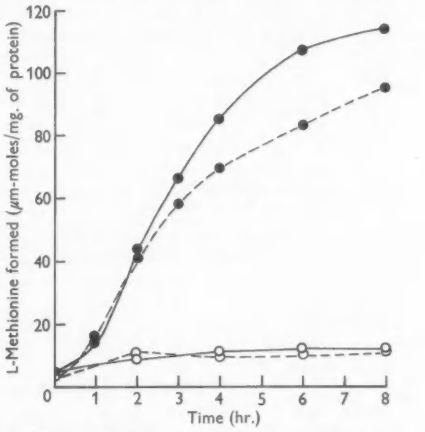

Fig. 2. Effect of cobalamin on the rate of formation of methionine by enzymic extracts of *E. coli* PA 15 in the presence of tetrahydropteroylglutamic acid (—) or its formaldehyde derivative (- - -). —, Reaction mixture *B* with enzymic extract and tetrahydro-PtGA (1 μmole). - - -, Reaction mixture *B* (without L-serine and pyridoxal phosphate and with DL-homocysteine decreased to 5 μ-moles) plus enzymic extract and tetrahydro-PtGA–CH_2O (3·3 μmoles). Cobalamin: present (70 $\mu\mu$moles) (●) or absent (○).

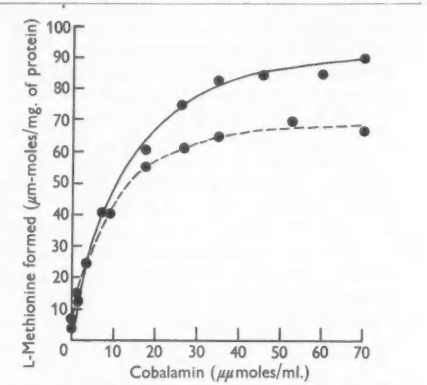

Fig. 3. Effect of the concentration of cobalamin on the synthesis of methionine by enzymic extracts of *E. coli* PA 15 in the presence of tetrahydropteroylglutamic acid or its formaldehyde derivative. Conditions for — (tetrahydro-PtGA present) and - - - (tetrahydro-PtGA–CH_2O present) were as described in Fig. 2. Incubated for 4 hr.

Table 4. *Requirement for cobalamin when the formaldehyde derivative of tetrahydropteroylglutamic acid replaces both folic acid and serine*

Strain 121/176: reaction mixture *A* (with DL-serine omitted) plus enzymic extract. Cobalamin (14 $\mu\mu$moles), tetrahydro-PtGA–CH_2O (3·6 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and DL-serine (20 μmoles when present). Incubated for 4 hr.

Strain PA15: reaction mixture *B* (with L-serine omitted) plus enzymic extract. Cobalamin (14 $\mu\mu$moles), tetrahydro-PtGA–CH_2O (3·3 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and L-serine (10 μmoles when present). Incubated for 4 hr.

Source of C_1 unit	Source of CoF	Cobalamin	L-Methionine formed (μm-moles/mg. of protein) Strain 121/176	Strain PA15
Serine	Extract of heated *E. coli*	Present	24	103‡
Tetrahydro-PtGA–CH_2O		Absent	6*	4
Tetrahydro-PtGA–CH_2O		Present	38†	40

* In four other experiments results ranged from 4 to 12.
† In four other experiments results ranged from 22 to 92.
‡ 101 when cobalamin was absent.

enzymic extracts of strain 121/176 and in Fig. 3 for those from strain PA15. The results were again variable in the sense described in the previous section for methionine synthesis in the presence of extract of heated *E. coli*.

Formaldehyde derivative of tetrahydropteroylglutamic acid. When tetrahydro-PtGA–CH_2O was used as source both of C_1 units and of folic acid coenzyme the synthesis of methionine by enzymic extracts of both strains again required cobalamin (Table 4, Fig. 2). The rate of methionine synthesis with strain PA15 was only slightly less than that when serine was donor and tetrahydro-PtGA the source of CoF (Fig. 2). The effect of increasing cobalamin concentrations was again variable with different enzyme preparations, but with the same extract similar results were obtained (Fig. 3) to those for the serine–tetrahydro-PtGA system.

Analogues and derivatives of cobalamin. These tests were carried out only with enzymic extracts of strain PA15 with serine as C_1-unit donor and tetrahydro-PtGA as source of folic acid coenzyme.

Coenzyme forms of vitamin B_{12} active in the metabolism of glutamate by *Clostridium tetanomorphum* have recently been described (Barker *et al.* 1958; Weissbach, Toohey & Barker, 1959). The 5:6-dimethylbenzimidazolylcobamide coenzyme was two to three times as active as cobalamin in the present test system (Fig. 4); the concentrations for half-maximal activation were 0·015 and 0·038 μM for the coenzyme and cobalamin respectively. Previous irradiation had no effect on the activity of cobalamin, but greatly decreased that of the coenzyme (Fig. 4). With the latter the effect of irradiation was apparently more marked at higher concentrations than at lower, suggesting the possibility that an inhibitor was formed.

A number of other compounds in which the

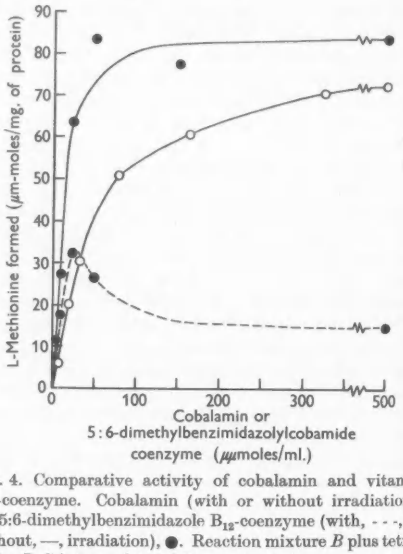

Fig. 4. Comparative activity of cobalamin and vitamin B_{12}-coenzyme. Cobalamin (with or without irradiation), ○; 5:6-dimethylbenzimidazole B_{12}-coenzyme (with, - - -, or without, —, irradiation), ●. Reaction mixture *B* plus tetrahydro-PtGA (1 μmole) and Dowex-treated enzymic extract of strain PA15. Irradiation of cobalamin and coenzyme was as described in the Materials and Methods section. Incubated for 3 hr.

nature of the base of the nucleotide residue differed from that in cobalamin was tested (Table 5); none had significantly higher activity than cobalamin itself at the concentrations tested. The substance with lowest activity was factor B (which has no nucleotide residue), but the further addition of 5:6-dimethylbenziminazole (itself inactive) resulted in activity equivalent to one-third of that of cobalamin. There was no quantitative correlation

with the relative growth-promoting activity of these compounds for the cobalamin auxotroph *E. coli* 113/3 as given by Kon & Pawelkiewicz (1958).

Presence of cobalamin during growth. In all the experiments so far described enzyme preparations were made from organisms grown in the absence of added cobalamin. Strain 121/176 requires either cobalamin or methionine for growth: when cobalamin at relatively low concentrations was added to the original growth medium an effect of cobalamin on methionine synthesis by the enzyme preparations was still obtained (Table 6). The effect was even significant at a concentration of cobalamin in the growth medium in excess of that required for optimum growth of the organism (0·7 $\mu\mu$mole/ml.). Results were similar when extract of heated *E. coli* served as source of folic acid coenzyme. Strain PA15 does not require cobalamin for growth, but its effect with enzymic extracts was not abolished unless the concentration in the growth medium exceeded 1·4 $\mu\mu$mole/ml. (Table 6).

It was also observed that increasing the concentration of cobalamin in the growth medium progressively increased the overall ability of the enzymic extracts of both organisms to synthesize methionine (Table 6); the rate of formation, however, decreased sharply after 2 hr. incubation.

Other strains of Escherichia coli. An effect of cobalamin with enzymic extracts of three other strains of *E. coli* was shown under appropriate conditions; all three systems were tested with regard to the nature of the donor of the C_1 unit and the source of folic acid coenzyme (Table 7). Strain 113/3 (like strain 121/176) is an auxotroph requiring cobalamin or methionine for growth; methionine synthesis by enzymic extracts was increased (as with strain 121/176) by cobalamin under all three conditions, though the effect was more marked when tetrahydro-PtGA rather than the extract of heated *E. coli* was the source of folic acid coenzyme. With the two prototrophic strains (518 and W) there was (as with strain PA15) no effect of cobalamin when extract of heated *E. coli* was used; however, with tetrahydro-PtGA present synthesis of methionine by enzymic extracts, though small, was markedly increased by the vitamin (Table 7).

Table 5. *Ability of analogues to replace cobalamin*

Reaction mixture *B* plus tetrahydro-PtGA (1 μmole) and enzymic extract of strain PA15. Incubated for 4 hr. Factor B, etc., indicate trivial names of the whole compound.

Nature of the base of the nucleotide residue	Amount of test compound ($\mu\mu$moles) 70	700
	Activity compared with cobalamin (%)	
No nucleotide present (factor B)	7	7
5-Methylbenzimidazole	5	45
Adenine (pseudovitamin B_{12})	30	100
2:6-Diaminopurine	55	80
5-Hydroxybenzimidazole (factor III)	85	90
2-Methyladenine (factor A)	100	95
5:6-Dichlorobenzimidazole	100	100
2-Methylmercaptoadenine	125	100

Effect of folic acid

The presence of some form of folic acid was necessary for appreciable synthesis of methionine by extracts of both organisms (strains 121/176 and PA15) under all test conditions investigated (Table 8); cobalamin did not affect the requirement. When tetrahydro-PtGA–CH_2O was used as donor of C_1 units the tetrahydro-PtGA residue was essential; replacement of tetrahydro-PtGA–CH_2O by the molar equivalent of formaldehyde decreased formation of methionine to low values (Table 8).

The effect of folic acid was studied in more detail with Dowex-treated extracts of strain PA15 with

Table 6. *Effect of growth in the presence of cobalamin on its subsequent activity with enzymic extracts*

Methionine was omitted from the growth medium for strain 121/176 except when no cobalamin was added. Reaction mixture *B* with enzymic extract of either strain 121/176 or strain PA15 and tetrahydro-PtGA (1 μmole). Cobalamin, 0·7 μm-mole when present. Incubated for 2 hr.

Cobalamin in growth medium ($\mu\mu$moles/ml.)	L-Methionine formed (μm-moles/mg. of protein) Strain 121/176 With cobalamin	Strain 121/176 Without cobalamin	Strain PA15 With cobalamin	Strain PA15 Without cobalamin
0	6*	67*	2	44
0·05	18	69	—	—
0·28	35	75	8	42
1·4	71	107	26	58
3·5	102	124	66	72
7·0	125	126	85	86
21·0	125	129	107	107

* Methionine was added to the growth medium.

serine as donor of C_1 units and tetrahydro-PtGA as source of folic acid (cobalamin present); the optimum concentration was about 0·5 μmole/ml. of reaction mixture (Fig. 5). The ability of extract of heated *E. coli* to promote synthesis of methionine in the absence of cobalamin was almost completely inhibited by tetrahydro-PtGA at concentrations as low as 0·1 μmole/ml. (Fig. 5). The same result was obtained with tetrahydro-PtGA freshly prepared from pteroylglutamic acid which had been purified by chromatography (Sakami & Knowles, 1959).

Effect of other factors

Substrates. Omission of either serine or homocysteine decreased synthesis of methionine to low values in all cases tested (Table 9); it is clear that the enzyme preparations used contained little of these amino acids. Serine could not be replaced as donor of C_1 units either by glycine (Table 9) or its formimino derivative; *N*-formylglycine and formate were also inactive with the one strain tested (PA 15). DL-Phosphoryl-*O*-serine had only 75 % of

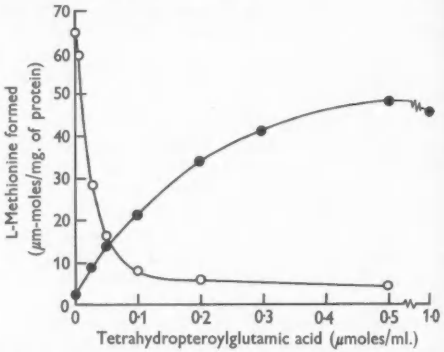

Fig. 5. Effect of the concentration of tetrahydropteroylglutamic acid on the synthesis of methionine by enzymic extracts of *E. coli* PA 15 in the presence of either cobalamin or extract of heated *E. coli*. Reaction mixture *B* with Dowex-treated enzymic extract supplemented with either 70 μμmoles of cobalamin (●) or with extract of heated *E. coli* (○). Incubated for 4 hr.

Table 7. *Effect of cobalamin on the synthesis of methionine by extracts of other strains of* Escherichia coli

Reaction mixture *A* plus enzymic extracts and with DL-serine omitted if tetrahydro-PtGA–CH_2O was present. Cobalamin (14 μμmoles), tetrahydro-PtGA (1 μmole) and tetrahydro-PtGA–CH_2O (3·6 μmoles) when present; extract of heated *E. coli* was used as described in the Materials and Methods section. Incubated for 4 hr. Cobalamin: +, present; −, absent.

		L-Methionine formed (μm-moles/mg. of protein)					
C_1-unit donor		Serine		Serine		Tetrahydro-PtGA–CH_2O	
Source of CoF		Extract of heated *E. coli*		Tetrahydro-PtGA			
Cobalamin		−	+	−	+	−	+
Strain of *E. coli*	113/3	30	39	5	14	9	31
	518	16	16	—	—	2	6
	W	26	27	4	20	5	8

Table 8. *Effect of folic acid on methionine synthesis by enzymic extracts of both strains of* Escherichia coli

Strain 121/176: reaction mixture *A* (DL-serine omitted when tetrahydro-PtGA–CH_2O was present) plus enzymic extract and cobalamin (14 μμmoles).

Strain PA 15: reaction mixture *B* (L-serine omitted when tetrahydro-PtGA–CH_2O was present) plus Dowex-treated enzymic extract and cobalamin (70 μμmoles). Other substances, when present: tetrahydro-PtGA (1 μmole), tetrahydro-PtGA–CH_2O (3·3 μmoles) and extract of heated *E. coli* (as in Materials and Methods section). Incubated for 4 hr.

Strain of *E. coli*	Conditions for methionine synthesis: C_1 donor	Conditions for methionine synthesis: Source of folic acid	L-Methionine formed (μm-moles/mg. of protein): With folic acid	L-Methionine formed (μm-moles/mg. of protein): Without folic acid
121/176	Serine	Extract of heated *E. coli*	5	24
PA 15	Serine	Extract of heated *E. coli*	4	76
PA 15	Serine	Tetrahydro-PtGA	7	70
121/176	Tetrahydro-PtGA–CH_2O		6*	38
PA 15	Tetrahydro-PtGA–CH_2O		4*	68

* For these experiments tetrahydro-PtGA–CH_2O was omitted and formaldehyde (3·3 μmoles) added.

Table 9. *Effect of other components of the reaction mixture on methionine synthesis by both strains under various conditions*

Conditions were as described for Table 8. Other substances when present: TPN (1 μmole), $DPNH_2$ (10 μmoles), extract of heated *E. coli* (as in Materials and Methods section) and glycine (10 μmoles). Incubated for 4 hr.

	L-Methionine formed (μm-moles/mg. of protein)				
Nature of C_1 donor	Serine		Serine		
Source of CoF	Extract of heated *E. coli*		Tetrahydro-PtGA	Tetrahydro-PtGA–CH_2O	
Strain of *E. coli*	121/176	PA15	PA15	PA15	121/176
Omission from reaction mixture					
None	24	76	70	68	38
ATP + Mg^{2+} ion	14	15	12	15	13
DPN	24	68	10	11	7
DPN (TPN added)	—	25	11	17	—
None (TPN added)	—	61	75	68	—
Glucose	—	39	10	15	11
Glucose + DPN	—	—	5	—	—
Glucose + DPN ($DPNH_2$ added)	—	—	63	—	—
DL-Homocysteine	3	4	5	8	—
DL- or L-Serine	4	8	7	—	—
DL- or L-Serine (glycine added)	5	11	—	—	—

the activity of DL-serine with strain 121/176; it may be hydrolysed before use. The tests with alternative potential C_1 donors were done with extract of heated *E. coli* as source of folic acid in case other and unknown cofactors were required.

Diphosphopyridine nucleotide. The requirement for DPN, which was clear with the other test systems, could not be shown with extract of heated *E. coli* present (Table 9); it presumably contains sufficient DPN. In the three conditions tested with strain PA15, TPN did not replace DPN and was somewhat inhibitory when extract of heated *E. coli* was the source of folic acid coenzyme (Table 9).

Both glucose and DPN could be effectively replaced by the single addition of $DPNH_2$ at high concentrations (Table 9; strain PA15, serine–tetrahydro-PtGA system); furthermore, synthesis of methionine reached about half the maximum even when ATP was omitted as well.

Adenosine triphosphate and magnesium ions. The effect of omitting these substances from the reaction mixture was more marked with strain PA15 than with strain 121/176, possibly due to the higher level of synthesis of methionine by the former strain.

Enzyme concentration. In the one case tested (strain PA15 with the serine–tetrahydro-PtGA system) synthesis of methionine increased linearly with concentration of enzymic extract up to the limit tested (10 mg. of protein/ml.).

Presence of methionine during growth. Wijesundera & Woods (1960) observed that growth in the presence of the amino acid markedly decreased its subsequent synthesis from homocysteine and serine by suspensions of a number of strains of *E. coli*. Increase of the concentration of methionine in the growth medium of strain 121/176 to mM, or addition of this concentration of the amino acid to the medium for strain PA15, resulted in a decrease in the activity of the enzymic extracts to about a quarter.

DISCUSSION

Effect of cobalamin. Kisliuk & Woods (1957, 1960), working with extracts of acetone-dried powders of *E. coli* PA15, found that tetrahydro-PtGA was only used in the transfer of C_1 units to homocysteine provided that the organism had been grown originally in the presence of cobalamin. The present results, some of which were obtained concurrently with the above work, provide a direct confirmation for a role of cobalamin in methionine synthesis since the free vitamin is active in promoting formation of methionine from homocysteine if the enzymic extracts are made by ultrasonic vibration rather than after drying with acetone. It is probable that treatment with acetone destroys some enzyme system or systems required for the further metabolism of cobalamin itself.

With enzymic extracts of strain PA15 there is no requirement for cobalamin when natural folic acid (provided as an extract of heated organisms) is the source of folic acid; cobalamin is needed, however, when tetrahydro-PtGA or its formaldehyde derivative is substituted for the natural folic acid. These results might suggest a function of cobalamin in the conversion of the simpler reduced forms of folic acid into a coenzyme form. On the other hand, ultrasonic extracts of strain 121/176

(a cobalamin auxotroph) still require cobalamin for the synthesis of methionine in the presence of heated extract of the same organism. The latter extract presumably contains an effective natural form of folic acid since it activates methionine synthesis by enzymic extracts of strain PA 15 in the absence of cobalamin. Extracts of heated suspensions of strain PA 15 did not allow methionine synthesis by the enzyme from strain 121/176 unless cobalamin was added; it is unlikely therefore that the heated extract contained any cobalamin (unpublished experiments of J. R. Guest). It is concluded provisionally that a role of cobalamin (if there is one) in the formation from tetrahydro-PtGA of a folic acid coenzyme cannot be the sole function of the vitamin in the overall methylation of homocysteine, at any rate by *E. coli* 121/176.

Other possibilities for the nature of the function of cobalamin, particularly that it (or a derivative) may act catalytically in the reduction of a compound containing a hydroxymethyl group to one with a methyl group, will not be examined here since they have been fully discussed by Kisliuk & Woods (1960). The present results do not add further to the arguments, but provide a firmer factual background.

The significantly higher activity (compared with cobalamin) of a form of vitamin B_{12} which has coenzyme activity in the metabolism of glutamate by *Clostridium tetanomorphum* (Barker *et al.* 1958; Weissbach *et al.* 1959) adds weight to the view that cobalamin must be transformed into a higher compound before it has functional activity in isolated enzyme systems. A few other analogues of vitamin B_{12} (with a different basic group in the nucleotide residue) had activity equal to or approaching that of cobalamin; it has not yet been determined whether they have intrinsic activity or whether they (and perhaps cobalamin also) are converted into one particular form by enzymes present in the crude extracts used.

Whatever the nature of the source of folic acid coenzyme or of the C_1-unit donor, the effect of increasing cobalamin concentration on methionine synthesis was quantitatively somewhat variable with different enzyme preparations. The overall reaction is undoubtedly complex and such variability may have been due to one or other of the enzymes present in the crude preparations used being in limiting supply in different batches.

Effect of other factors. Tetrahydropteroylglutamic acid almost completely inhibited the synthesis of methionine promoted by ultrasonic extracts of strain PA 15 in the presence of extract of heated organisms (no cobalamin added). This is similar to the effect described and discussed by Kisliuk & Woods (1960), where the activity of natural folic acid coenzyme in extracts of acetone-dried powders not treated with Dowex-1 resin was severely inhibited by tetrahydro-PtGA. No factual explanation has so far been obtained.

A requirement for DPN in the overall reaction showed up clearly in the test systems not containing extract of heated organisms; the latter presumably contains this factor. Both glucose and DPN could be replaced by $DPNH_2$ at concentrations of the same order as those of serine and homocysteine; the main function of glucose is therefore probably in the generation of $DPNH_2$. The reduced form of the coenzyme was not, however, used as a routine since the enzymic extracts had strong $DPNH_2$-oxidase activity. In their enzyme system derived from a cobalamin auxotroph of *E. coli*, Hatch, Takeyama, Cathou, Larrabee & Buchanan (1959) have found that $DPNH_2$ can be replaced (under an atmosphere of hydrogen) by reduced flavin mononucleotide or reduced flavinadenine dinucleotide.

It is clear that glucose, DPN and ATP + Mg^{2+} ions are necessary for the acceptance by homocysteine of the C_1 unit and its reduction to a methyl group; these substances were still required when serine was replaced as donor by tetrahydro-PtGA–CH_2O.

With enzyme systems isolated from animal tissues for the methylation of homocysteine, TPN is more effective than DPN (Nakao & Greenberg, 1958; Stevens & Sakami, 1959). With enzymic extracts of strain PA 15, TPN was inhibitory when the extract of heated *E. coli* was source of folic acid and did not replace DPN (though it was not inhibitory) in the two test systems in which tetrahydro-PtGA or its formaldehyde derivative served (in the presence of cobalamin) as coenzyme. Kisliuk & Woods (1960), using enzyme preparations from acetone-dried organisms, have also found that TPN does not replace DPN and is inhibitory.

SUMMARY

1. Ultrasonic extracts of *Escherichia coli* strain 121/176 (a cobalamin or methionine auxotroph) and of strain PA 15 (a serine or glycine auxotroph) synthesize methionine from homocysteine and serine under certain conditions in the presence of glucose, adenosine triphosphate, magnesium ions, diphosphopyridine nucleotide and a source of natural folic acid (extract of heated organism).

2. With strain 121/176 the further provision of cobalamin is necessary if the organism has been grown originally on a medium devoid of this vitamin. Natural folic acid can be replaced by tetrahydropteroylglutamate.

3. With strain PA 15 cobalamin is not required if the natural source of folic acid coenzyme is

provided. Tetrahydropteroylglutamate can, however, only be used if cobalamin is present.

4. The formaldehyde derivative of tetrahydropteroylglutamate can serve with both strains both as source of folic acid coenzyme and of C_1 units provided again that cobalamin is present.

5. Growth of either strain in the presence of cobalamin enhances the ability of ultrasonic extracts of the organisms to methylate homocysteine.

6. Reduced diphosphopyridine nucleotide abolishes the requirement for both glucose and diphosphopyridine nucleotide.

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Inhibition of Methionine Synthesis in *Escherichia coli* by Analogues of Cobalamin

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A relationship between cobalamin and the synthesis of methionine by *Escherichia coli* was first established by the isolation of auxotrophic strains requiring either cobalamin or methionine for growth (Davis & Mingioli, 1950). Cobalamin has since been found to increase methionine synthesis from homocysteine and serine by washed suspensions of many strains of *E. coli* (Gibson & Woods, 1960); such synthesis is also increased several-fold by the addition of cobalamin to ultrasonic extracts of an auxotrophic strain, *E. coli* 121/176, which requires cobalamin or methionine for growth (Helleiner & Woods, 1956). More recently ultrasonic extracts of another auxotrophic strain (PA 15), which does not require cobalamin for growth, have been found to require the vitamin for methionine synthesis only when the source of the cofactor for the transfer of the C_1 unit is tetrahydropteroylglutamic acid and not when heated extract of the organism is used. With ultrasonic extracts of strain 121/176, cobalamin is essential with both sources of the folic acid cofactor (Helleiner, Kisliuk & Woods, 1957; Guest, Helleiner, Cross & Woods, 1960).

Several analogues of cobalamin have been prepared (Smith, Parker & Gant, 1956) and reported

to inhibit the growth of the cobalamin-requiring auxotroph *E. coli* 113/3 (Cuthbertson, Gregory, O'Sullivan & Pegler, 1956). Baker *et al.* (1959), however, have not found them to be active in this respect. These analogues have also been shown to inhibit a possible function of cobalamin in amino acid activation and protein synthesis (Wagle, Mehta & Johnson, 1958*a*, *b*); however, a direct action of cobalamin here has not been confirmed by Fraser & Holdsworth (1959) or by Arnstein & Simkin (1959). Recently the analogues have been found to inhibit the activity of a cobalamin-containing enzyme of *E. coli* required for methionine formation from serine and homocysteine (Hatch, Takeyama & Buchanan, 1959).

In the present work the effect of analogues and derivatives of cobalamin on the synthesis of methionine by ultrasonic extracts of *E. coli* has been studied in an attempt to elucidate further the role of cobalamin.

MATERIALS AND METHODS

Organisms and extracts. Stock cultures of *E. coli* were maintained on slopes of tryptic meat–agar, subcultured monthly, incubated for 16 hr. at 37° and stored at 4°. Four strains have been used; two of these were auxotrophic strains (121/176 and 113/3), which require cobalamin or methionine for growth (Davis & Mingioli, 1950), and another was their parent (strain W). The greater part of the work, however, was with strain PA 15, which requires serine or glycine for growth and was originally obtained from Dr Barbara Wright.

The growth medium and the preparation of the ultrasonic extract have been described by Guest *et al.* (1960). The medium was supplemented with glycine (10 mM) for strain PA 15 and DL-methionine (0·5 mM) for strain 121/176. In a few experiments cobalamin or its analogues were added to the complete medium for strain PA 15; they were sterilized separately by Seitz-filtration.

Extract of heated organisms was prepared by heating a washed suspension (170 mg. dry wt./ml.) at 100° for 3 min., cooling and centrifuging. The supernatant fluid will be referred to as extract of heated *E. coli*, and is a source of natural folic acid coenzyme.

Study of methionine synthesis. The solution used for testing methionine synthesis was similar to reaction mixture *B* of Guest *et al.* (1960) and contained (in 2 ml. of 0·125 M-potassium phosphate buffer, pH 7·8): L-serine, 10 μmoles; DL-homocysteine, 10 μmoles; glucose, 20 μmoles; $MgCl_2$, 10 μmoles; adenosine triphosphate (ATP), 10 μmoles; diphosphopyridine nucleotide (DPN), 1 μmole; pyridoxal phosphate, 0·5 μmole. In experiments with the formaldehyde derivative of tetrahydropteroylglutamic acid (tetrahydro-PtGA–CH_2O) the amount of DL-homocysteine was decreased to 5 μmoles and pyridoxal phosphate and serine were omitted (Kisliuk & Woods, 1960).

When extract of heated *E. coli* was added the amount was equivalent to 17 mg. dry wt. of original organisms. The volume of ultrasonic extract used in all experiments contained 10 mg. of protein; the latter was estimated spectrophotometrically according to Layne (1957).

The complete reaction mixture was incubated at 37° for 4 hr. under an atmosphere of H_2. Methionine synthesis was stopped by heating at 100° for 3 min. After centrifuging to remove precipitated protein the supernatant fluid was assayed for methionine with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P 60 (Gibson & Woods, 1960). Presence in the basal medium of cobalamin (or of any of its analogues used in this work) at concentrations equivalent to those in the samples of reaction mixture to be assayed, had no effect on the response of the organism to methionine.

The amount of L-methionine formed is given as μm-moles/mg. of protein of the ultrasonic extract. The value obtained for a control, containing all components of the reaction mixture but heated at zero time, was subtracted from the experimental and other control values.

Experiments with growing cultures. Liquid-culture technique was adopted for studying the growth of strains of *E. coli* in the presence of cobalamin and its analogues. The medium described previously (Guest *et al.* 1960) was used at half the final concentration of all constituents. Duplicate tests were carried out in 150 mm. × 20 mm. hard-glass tubes containing, with the appropriate supplements and inoculum, a final volume of 2 ml. Solutions of cobalamin and its analogues were sterilized by Seitz-filtration and added to the medium, which had been autoclaved at 115° for 10 min.

The inoculum was prepared from a tryptic meat–agar-slope culture of the organism and harvested after incubation for 16 hr. at 37°. The organisms were washed once with 10 ml. of water and each tube of the tests was inoculated with about 3·5 μg. dry wt. of organisms (in 0·1 ml.). Incubation was for 24 or 48 hr. at 37° with the tubes sloped to increase aeration.

The extent of growth was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) with a neutral-density filter; the uninoculated medium was used to give the zero setting. With high concentrations of cobalamin analogues, the value for an uninoculated control was deducted from the corresponding experimental values. The instrument was calibrated in terms of mg. dry wt. of organisms/ml. of culture and results are expressed in this way. The inhibition index for half-maximal and complete inhibition of growth is defined as the ratio, concentration of analogue : concentration of cobalamin.

Chemicals. The source of cobalamin was Anacobin (British Drug Houses Ltd., Poole, Dorset); it was assayed spectrophotometrically with the extinction coefficients quoted by Brink *et al.* (1949). The methylamide, ethylamide and anilide of the monocarboxylic acids of cyanocobalamin (Smith *et al.* 1956) were provided by Dr E. Lester Smith; they will be referred to as the methylamide, ethylamide and anilide analogues. They contained less than 0·02 % of cobalamin itself (E. L. Smith, personal communication). Crystalline samples were dissolved in water and examined spectrophotometrically in visible and ultraviolet light. The spectra were identical with cobalamin except that the anilide analogue had a higher absorption in the 220–275 mμ region, owing to the presence of one additional benzene ring in the molecule. The molecular extinction coefficients at the three wavelengths of maxi-

mum absorption (278, 361, 550 mμ) were assumed to be the same as those of cobalamin.

Factor B, which has no nucleotide residue, was also provided by Dr E. Lester Smith and was assayed spectrophotometrically by assuming that the molecular extinction coefficient in 0·01% KCN solution at the absorption maximum (367 mμ) is the same as for cobalamin under the same conditions.

A specimen of 5:6-dimethylbenzimidazole B_{12}-coenzyme was given by Dr H. A. Barker and it was also assayed spectrophotometrically (Weissbach, Toohey & Barker, 1959).

The values given for the concentration of cobalamin and analogues have been corrected for their probable purities, which were: cobalamin, 88%; 5:6-dimethylbenzimidazole B_{12}-coenzyme, 100%; methylamide, 82%; ethylamide, 84%; anilide, 83% and factor B, 70%.

Other chemicals and their sources were as described by Guest *et al.* (1960).

RESULTS

Methionine synthesis by cell-free extracts

The synthesis of methionine from homocysteine by ultrasonic extracts of strains of *E. coli* was studied under three conditions: (I) serine as the donor of the C_1 unit and tetrahydropteroylglutamic acid (tetrahydro-PtGA) as the folic acid cofactor; (II) the formaldehyde derivative of tetrahydro-PtGA (tetrahydro-PtGA–CH_2O) as both donor of the C_1 unit and as the cofactor; (III) serine as donor and extract of heated *E. coli* as source of folic acid cofactor. With ultrasonic extracts of strain 121/176, cobalamin is required in every case, but, for strain PA 15, cobalamin is only essential when tetrahydro-PtGA is the cofactor for transferring C_1 units (i.e. conditions I and II).

Factor B and the methylamide, ethylamide and anilide derivatives of the monocarboxylic acids of cobalamin were tested for their ability to replace or inhibit the action of cobalamin in these three conditions for methionine synthesis.

Methionine synthesis in the presence of serine and tetrahydropteroylglutamic acid. The analogues of cobalamin replace the requirement for cobalamin to a limited extent, but only at relatively high concentrations (Table 1). The analogues, however, were also inhibitors of the action of cobalamin and decreased the synthesis of methionine to the value obtained with the analogue alone. The order of increasing activity in this respect was methylamide < factor B < ethylamide < anilide (Table 1). The two extreme cases of the analogue activities are illustrated in Fig. 1. Because the analogues have some ability to replace cobalamin, and because this is different for each analogue, it is not possible to express their capacities for inhibition in precise quantitative terms. The anilide analogue was chosen for more detailed studies because it combines high inhibitory activity with a weak ability to replace the vitamin.

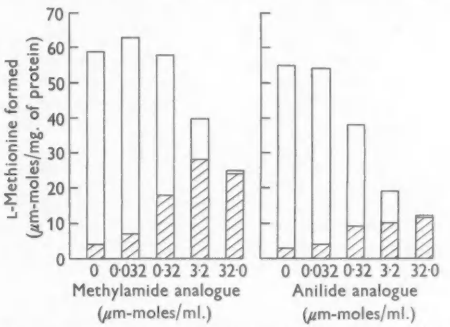

Fig. 1. Effect of the methylamide and anilide analogues of cobalamin on methionine synthesis. Reaction mixture plus ultrasonic extract of strain PA 15 and tetrahydro-PtGA (1 μmole). Cobalamin: □, present (0·032 μm-mole/ml.); ▨, absent. Incubated for 4 hr.

Table 1. *Effect of analogues of cobalamin on methionine synthesis by ultrasonic extract of* Escherichia coli *in a system containing serine and tetrahydropteroylglutamic acid*

Reaction mixture (see Materials and Methods section) plus ultrasonic extract and tetrahydro-PtGA (1 μmole); cobalamin, 0·032 μm-mole/ml. when present. Incubated for 4 hr.

		Cobalamin absent					Cobalamin present				
		Concn. of analogue (μm-moles/ml.)									
Strain of *E. coli*	Analogue	0	0·032	0·32	3·2	32·0	0	0·032	0·32	3·2	32·0
		L-Methionine formed (μm-moles/mg. of protein)									
PA 15	Methylamide	4	7	18	28	24	59	63	58	40	25
	Ethylamide	5	7	13	20	21	73	72	63	33	22
	Anilide	3	4	9	10	12	55	54	38	19	12
	Factor B	3	5	7	8	9	62	61	59	24	11
121/176	Anilide	2	3	5	7	8	68	68	34	10	8

Treatment of the enzymic extract with Dowex-1 resin as described by Kisliuk & Woods (1960) had no effect on the inhibitory action of the anilide analogue.

In order to determine the type of inhibition, the rate of methionine formation was measured over a range of cobalamin concentrations (0–0·32 μm-moles/ml.) in the presence of two concentrations of the anilide analogue (0·73 and 1·45 μm-moles/ml.). The results were plotted by the method of Lineweaver & Burk (1934) and show (Fig. 2) that the anilide analogue is a competitive inhibitor of cobalamin. A second experiment of the same type with different concentrations of the anilide (0·29 and 2·9 μm-moles/ml.) confirmed this conclusion.

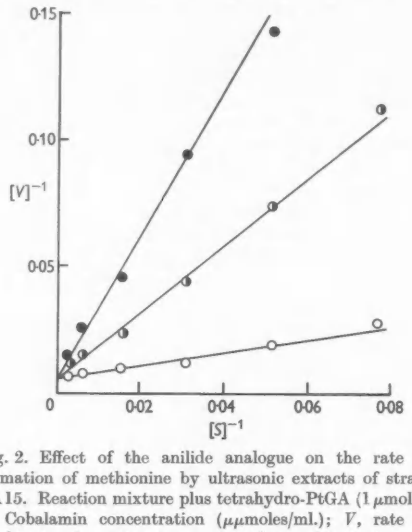

Fig. 2. Effect of the anilide analogue on the rate of formation of methionine by ultrasonic extracts of strain PA 15. Reaction mixture plus tetrahydro-PtGA (1 μmole). *S*, Cobalamin concentration (μμmoles/ml.); *V*, rate of methionine formation (μm-moles of L-methionine/mg. of protein/4 hr.). Anilide analogue: ○, nil; ◑, 0·73 μm-mole/ml.; ●, 1·45 μm-mole/ml.

Table 2. *Effect of analogues of cobalamin on methionine synthesis by ultrasonic extract of* Escherichia coli *in a system containing the formaldehyde derivative of tetrahydropteroylglutamic acid*

Reaction mixture (modified as described in the Materials and Methods section) plus enzymic extract of strain PA 15 and tetrahydro-PtGA–CH_2O, (3·3 μmoles); cobalamin, 0·032 μm-mole/ml. when present. Incubated for 4 hr.

Analogue		L-Methionine formed (μm-moles/mg. of protein)	
Nature	Concn. (μm-moles/ml.)	Cobalamin absent	Cobalamin present
None	—	6	62
Methylamide	3·0	34	44
	30·0	28	32
Ethylamide	1·0	21	51
	30·0	24	25
Anilide	0·5	12	53
	29·0	19	24
Factor B	1·7	7	36
	34·5	8	10

Table 3. *Effect of analogues of cobalamin on synthesis of methionine by ultrasonic extract of* Escherichia coli *in a system containing serine and heated extract of* Escherichia coli

Reaction mixture plus Dowex-treated ultrasonic extract and heated extract of the corresponding strain (see Materials and Methods section). Cobalamin, 0·032 μm-mole/ml. when present. Incubated for 2 hr. (strain PA 15) or for 4 hr. (strain 121/176).

Strain of *E. coli*	Analogue		L-Methionine formed (μm-moles/mg. of protein)	
	Nature	Concn. (μm-moles/ml.)	Cobalamin absent	Cobalamin present
PA 15	Methylamide	0	54	—
		3·0	59	—
		30·0	57	—
	Ethylamide	0	54	—
		1·0	57	—
		30·0	60	—
	Anilide	0	67	70
		0·5	71	71
		29·0	70	74
	Factor B	0	54	60
		1·7	58	58
		34·5	56	56
121/176	Anilide	0	5	37
		0·5	7	18
		29·0	10	10

Methionine synthesis in the presence of the formaldehyde derivative of tetrahydropteroylglutamic acid. Two concentrations of analogue only were used for testing their activity when serine and tetrahydro-PtGA were replaced by tetrahydro-PtGA–CH_2O. These were those giving half and maximum inhibition under condition I. The results were essentially the same as those for condition I both in replacing cobalamin and in inhibiting its action (Table 2).

Methionine synthesis in the presence of serine and extract of heated E. coli. The synthesis of methionine by ultrasonic extracts of strain PA15 with extract of heated *E. coli* as source of folic acid coenzyme was not inhibited by the analogues whether or not cobalamin was added (Table 3). With enzymic extracts of strain 121/176 and extract of heated *E. coli*, methionine synthesis occurred only if cobalamin was added, and here the anilide analogue was inhibitory; the degree of inhibition was the same whether the source of folic acid cofactor was the heated extract or tetrahydro-PtGA.

These results suggest that if cobalamin is present in the heated extract of strain PA15 it is not in a form that can function in the synthesis of methionine. However, there may be a more active form of cobalamin whose action is not inhibited by the anilide analogue. This possibility was investigated by interchanging the enzymic and heated extracts of the two strains of *E. coli* (Fig. 3). Dialysed and Dowex-treated extracts of strain PA15 showed no requirement for cobalamin if the source of heated extract was prepared from either strain PA15 or strain 121/176, whereas similar enzymic extracts of strain 121/176 required cobalamin even if the heated extract was derived from strain PA15. In addition, the synthesis of methionine by enzymic extracts of strain PA15 with heated extract derived from strain 121/176 was not affected (either in the presence or absence of cobalamin) by concentrations of the anilide analogue which were inhibitory in systems in which cobalamin is required.

Effect of the anilide analogue upon methionine synthesis when cobalamin is replaced by 5:6-dimethylbenzimidazole B_{12}-coenzyme. Under condition I (serine, tetrahydro-PtGA system) 5:6-dimethylbenzimidazole B_{12}-coenzyme, a factor required for the metabolism of glutamate by *Clostridium tetanomorphum* (Barker, Weissbach & Smyth, 1958; Weissbach *et al.* 1959), is two to three times as active as cobalamin in promoting the synthesis of methionine (Guest *et al.* 1960). Much higher concentrations of the anilide analogue were required to inhibit the action of the B_{12}-coenzyme than those effective with an equimolar concentration of cobalamin (Fig. 4). In order to determine the type of inhibition here, two concentrations of B_{12}-coenzyme, one three times as high as the other, were chosen, and the effect of a series of concentrations of the anilide analogue was studied. The percentage inhibition of the action of B_{12}-coenzyme was constant for a constant ratio of anilide to B_{12}-coenzyme concentration (Table 4). The inhibition is therefore, as with cobalamin, of the competitive type over the range of concentrations tested.

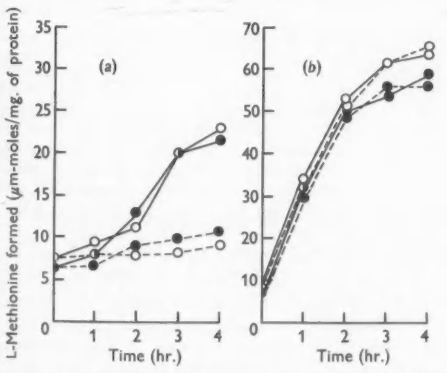

Fig. 3. Activity of heated extracts of strains of *Escherichia coli*. Progress of methionine formation by ultrasonic extracts of (*a*) strain 121/176 and (*b*) strain PA15. Reaction mixture plus Dowex-treated enzymic extract with: ○, heated extract of strain 121/176; ●, heated extract of strain PA15; cobalamin: —, present (32 μμmoles/ml.); - - -, absent.

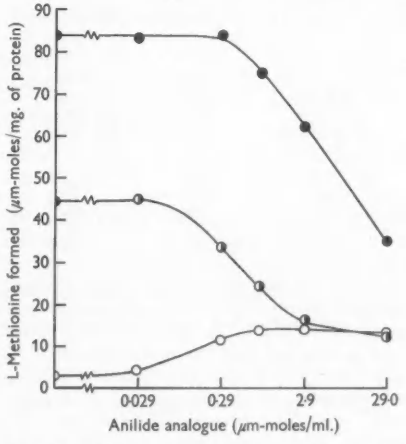

Fig. 4. Inhibition by the anilide analogue of methionine synthesis in the presence of cobalamin and 5:6-dimethylbenzimidazole B_{12}-coenzyme. Reaction mixture plus Dowex-treated extract of strain PA15 and tetrahydro-PtGA (1 μmole); ○, No addition; ◑, cobalamin (32 μμmoles/ml.); ●, 5:6-dimethylbenzimidazole B_{12}-coenzyme (32 μμmoles/ml.). Incubated for 4 hr.

Effect of the anilide analogue upon methionine synthesis by extracts of organisms grown in the presence of cobalamin. When cobalamin (4 $\mu\mu$moles/ml.) is added to the growth medium for strain PA15, the ability of enzymic extracts to synthesize methionine without added cobalamin becomes just limiting (Guest *et al.* 1960). Under these conditions the anilide analogue, even at 29 μm-moles/ml., had no inhibitory action. If the anilide was added instead to the growth medium together with cobalamin (4 $\mu\mu$moles/ml.), the activity of the enzymic extract obtained was decreased (Fig. 5). Considerable activity, however, was present in extracts of organisms harvested after growth in the presence of the anilide only. When the source of folic acid coenzyme was extract of heated *E. coli*, enzymic extracts of organisms derived from media containing the anilide analogue with or without added cobalamin had the same activity for methionine formation.

Anilide analogue and growth of Escherichia coli

Over the range of concentrations tested (0·029–29·0 μm-moles/ml.), the anilide analogue did not support the growth of strains requiring cobalamin or methionine, nor did it inhibit growth when an optimum concentration of methionine (0·5 mM) was present. The anilide analogue had little effect on the growth of strain PA15 in the presence of glycine (5 mM) or of strain W.

When cobalamin was added to the basal medium at a concentration greater than optimum for the growth of strain 121/176, the anilide analogue inhibited the growth of strains 121/176 and 113/3 (Table 5). The inhibition indices for 50% inhibition of growth at 24 hr. at three concentrations of cobalamin (0·13, 0·65 and 6·5 $\mu\mu$moles/ml.) were 550, 570 and 370 for strain 121/176 and 550, 580 and 390 for strain 113/3. For full inhibition the values were about 1000 for both strains in every case. For strains PA15 and W, which do not

Table 4. *Inhibition by the anilide analogue of the synthesis of methionine promoted by 5:6-dimethylbenzimidazole* B_{12}*-coenzyme by ultrasonic extract of* Escherichia coli

Reaction mixture plus ultrasonic extract of strain PA15 and tetrahydro-PtGA (1 μmole). Incubated for 4 hr. in the dark.

Anilide analogue (μm-moles/ml.)	B_{12}-coenzyme (μm-mole/ml.) 0·011	0·032
	Inhibition (%) of the action of B_{12}-coenzyme in methionine synthesis	
0	0	0
0·29	20	0
0·87	40	23
2·61	54	38
7·83	70	56
23·49	—	73

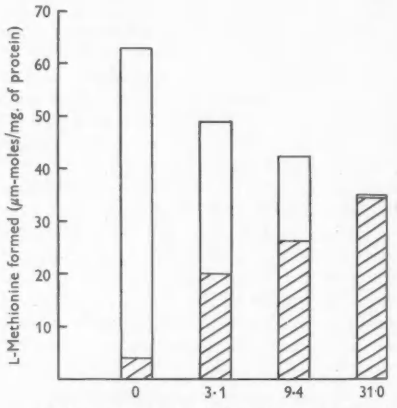

Fig. 5. Effect of growing *Escherichia coli* PA15 in the presence of the anilide analogue on the ability of ultrasonic extracts to synthesize methionine. Reaction mixture plus tetrahydro-PtGA (1 μmole) and extract of organisms cultured with cobalamin (□, 4 $\mu\mu$moles/ml. of growth medium) or without cobalamin (▨). Incubated for 1 hr. 40 min.

Table 5. *Effect of the anilide analogue on the growth of* Escherichia coli

Basal medium was supplemented with glycine (5 mM) and cobalamin (0·65 $\mu\mu$mole/ml.) where stated.

Strain of *E. coli*	Additions to basal medium	Time of incubation (hr.)	Anilide analogue (μm-moles/ml.) 0	0·029	0·29	2·9	29·0
			Growth (mg. dry wt. of organisms/ml.)				
W	—	24	0·7	0·7	0·7	0·8	0·9
	Cobalamin	24	1·2	1·1	0·7	0·7	0·7
	Cobalamin	48	2·5	2·5	2·6	2·6	2·8
PA15	Glycine	24	0·4	0·4	0·4	0·4	0·4
	Glycine, cobalamin	24	0·5	0·5	0·4	0·3	0·3
	Glycine, cobalamin	48	1·7	1·7	1·6	1·7	1·7
121/176	Cobalamin	24	0·5	0·5	0·3	0·0	0·0
113/3	Cobalamin	24	0·6	0·6	0·4	0·0	0·0

depend on added cobalamin for growth, addition of the vitamin increased the early growth (Table 5). This effect was more pronounced after 16 hr. and was still apparent after 24 hr., but cobalamin had no effect on the extent of growth at 48 hr. The anilide analogue (at concentrations which inhibited the growth of strains 121/176 and 113/3) also inhibited this increase in early growth, but it had no effect on the extent of growth at 48 hr.

DISCUSSION

Synthesis of methionine is inhibited by the analogues of cobalamin whenever cobalamin is essential for such synthesis. The anilide analogue shows a competitive relationship with cobalamin and the inhibitory activity is thus related specifically to the function of cobalamin. The ability of the analogues both to replace cobalamin and inhibit its action makes it difficult to interpret the results in a quantitative manner. There are several possible explanations for this phenomenon. First, the analogues may have sufficient structural similarity to cobalamin to function as cobalamin at low efficiency. Secondly, the enzymic extracts may convert a proportion of the analogue into cobalamin. The contamination of the analogue with cobalamin is less than 0·02 % (E. L. Smith, personal communication) and this would not account for their ability to replace cobalamin.

Escherichia coli 113/3, which requires cobalamin for growth, can grow in the presence of factor B, which is converted into complete cobalamins (Ford & Porter, 1953; Ford, Holdsworth & Kon, 1955). Factor B in the presence of 5:6-dimethylbenzimidazole is weakly active in methionine synthesis with extracts of *E.* coli strain PA 15 (Guest *et al.* 1960). However, alone it had low activity in replacing cobalamin whereas at high concentrations it proved to be an active inhibitor of the action of cobalamin.

A coenzyme form of cobalamin, 5:6-dimethylbenzimidazole B_{12}-coenzyme, which is more active than cobalamin in the synthesis of methionine, was found to require much higher concentrations of the anilide analogue to inhibit its activity. It is possible that cobalamin may first be transformed into such a compound, but if this is so it is unlikely to be the ultimate functional material in the synthesis of methionine since (*a*) inhibition of the activity of the coenzyme by the anilide analogue is still competitive in nature and (*b*) extracts of strain PA 15 cultured in the presence of cobalamin can synthesize methionine and this is insensitive to the anilide.

The ability of enzymic extracts of strain PA 15, grown in the presence of cobalamin, to synthesize methionine was decreased if high concentrations of the anilide analogue were also added to the growth medium. The relative concentrations of the anilide to cobalamin which produce this effect were of a similar order to those which inhibit the growth of cobalamin-requiring strains. However, the inhibition is not absolute because the extracts of organisms grown in the presence of the anilide with no added cobalamin have considerable activity. On the data available for the upper limit of possible contamination of the anilide analogue with cobalamin this could be due to such contamination; it becomes a factor of possible significance with the high concentrations of analogue relative to cobalamin used in these experiments.

Two experiments indicate that cobalamin is not present in the heated extract of *E. coli* PA 15. The anilide analogue did not inhibit the synthesis of methionine by enzymic extracts of strain PA 15 when extract of heated *E. coli* was present. Furthermore, heated extract derived from strain PA 15 did not alone promote methionine synthesis by enzymic extracts of the cobalamin auxotroph, strain 121/176, which requires cobalamin for this purpose.

The ability of the anilide analogue to inhibit the growth of strains dependent upon cobalamin for growth is in agreement with the findings of Cuthbertson *et al.* (1956). The critical ratio, concentration of anilide : concentration of cobalamin, is higher for growth than for the synthesis of methionine by enzymic extracts. This may reflect differences in the permeability of cobalamin and the analogue for the whole organism, a higher external concentration of analogue being needed to achieve an inhibitory concentration at the site of cobalamin action.

SUMMARY

1. Under certain conditions cobalamin is required for the synthesis of methionine by ultrasonic extracts of strains of *Escherichia coli*. The action of cobalamin is inhibited by the methylamide, ethylamide and anilide of the monocarboxylic acids of cobalamin and by factor B.

2. The anilide analogue inhibits competitively both the action of cobalamin and of 5:6-dimethylbenzimidazole B_{12}-coenzyme, but is less active against the latter.

3. When an extract of heated oranisms is used as source of folic acid coenzyme with enzymic extracts of a strain not requiring cobalamin for growth, cobalamin is not required for methionine synthesis and the analogues are not inhibitory. Extracts of organisms grown in the presence of cobalamin do not require added cobalamin for methionine synthesis and the anilide analogue again does not inhibit.

4. The anilide analogue inhibits the growth of strains requiring cobalamin or methionine for

growth when cobalamin is the growth factor, but not when methionine is supplied. It does not inhibit the growth of strains which do not require cobalamin for growth.

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Chemical Changes in Rabbit Aorta and Ear Cartilage Induced by Papain and Calciferol

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It has been shown both histochemically (Thomas, 1956) and chemically (Tsaltas, 1958) that the intravenous administration of a solution of crude papain decreases the mucopolysaccharide content of rabbit-ear cartilage.

With regard to the action of papain *in vivo*, it has been shown by McCluskey & Thomas (1958) that the injection of crystalline papain results in depletion of cartilage matrix only if the enzyme is inactivated by oxidation or sulphydryl blocking agents before administration. Cysteine-activated crystalline papain produces little effect on cartilage when injected. The changes produced by injection of inactivated crystalline papain were found to be indistinguishable from those produced by crude papain. These workers suggested that the reason for the failure of activated papain to enter cartilage after intravenous injection is that it probably reacts with one or more substrates present in blood. Oxidized or inactivated papain, on the other hand, appears to be taken up by cartilage and converted into the active form. In the present study a solution of crude non-activated papain (L. Light and Co. Ltd) in 0·9% sodium chloride solution was used.

It has also been shown that after toxic doses of calciferol in rats, there is an increase in total hexose and hexosamine content of the aorta, which parallels the aortic mineralization which occurs (Gillman, Grant & Hathorn, 1960). Because of the possibility that mucopolysaccharides may play an important role in the process of calcification, it was decided to test the effects of papain on the action of calciferol given simultaneously.

METHODS

Four young hybrid rabbits (three male and one female) with a mean weight of 1960±70 g. (S.E.) were randomly allocated to each of the following treatment groups (a total of 16 rabbits being employed): group I, these were injected intravenously in the left ear with a 1% solution of crude papain (L. Light and Co. Ltd.) in 0·9% NaCl solution (1 ml./kg. body wt.) on the first, fourth, seventh and tenth days of the experiment; group II, as in group I, but injected with 0·9% NaCl solution instead of papain solution; group III, papain injections were given as in group I, together with 0·5 mg. of calciferol (Petersen and Co., Cape Town)/kg. body wt. given orally daily for 10 days as a solution in arachis oil (15 mg./ml.); group IV, these were given injections of 0·9% NaCl solution as in group II together with calciferol as in group III.

The rabbits were killed after 10 days, and the cartilage of the right ear and the entire aorta was removed for chemical analysis. Freshly excised tissues were freed from connective tissue, rinsed in 0·9% NaCl solution, cut into small pieces and placed under dry acetone. After 24 hr. the acetone was renewed and allowed to stand for a further 24 hr. period. The acetone was then replaced with a mixture of ether and ethanol (1:1, v/v) and after standing overnight the solvent was removed and the tissues were dried to constant weight in a hot-air oven at 100°.

Hydrolysis. Portions of dry tissue (50 mg.) were heated with 4 ml. of 0·5 N-HCl for 4 hr. at 100° in sealed tubes. The solutions were then filtered and the volumes adjusted to 10 ml.

Hexoses. These were determined in 1 ml. portions of the hydrolysate with anthrone according to the method of Trevelyan & Harrison (1952), the results being expressed as glucose. Tests with glucose showed that the amount of destruction during the hydrolysis period was insignificant. The yield of hexose as determined by anthrone appeared to be maximal under these conditions.

Hexosamines. These were determined by a modification of the Elson & Morgan method (1933). The acetylacetone reagent was made by dissolving 1 ml. of redistilled acetylacetone in 25 ml. of M-Na_2CO_3 (A.R.) solution. To 1 ml. of hydrolysate 2 ml. of reagent was added and the mixture heated for exactly 15 min. in a boiling-water bath. After cooling, 6 ml. of diluted reagent [consisting of 1·6 g. of *p*-dimethylaminobenzaldehyde (Merck) dissolved in a mixture of 30 ml. of redistilled ethanol and 30 ml. of conc. HCl and diluted with 300 ml. of ethanol] was added. The tube was allowed to stand at room temperature for 10 min. with occasional shaking to remove CO_2 and the colour density of the solution was measured at 530 mμ in a Zeiss spectrophotometer, model P.M. QII. Each run included a duplicate standard (50 μg. of glucosamine). Tests with finely divided, acetone-dried and defatted aorta and cartilage and also with chondroitin sulphate showed that the hydrolysis conditions resulted in a maximum yield of hexosamine as determined by the Elson–Morgan reaction. Also, examination of the absorption spectra of the reaction products between 450 and 600 mμ showed a single well-defined peak with a maximum at 530 mμ, closely resembling the curve obtained with pure glucosamine. According to Boas (1953) this indicates that only small amounts of interfering chromogens are present, and it was not therefore considered necessary to separate the amino sugars by means of ion-exchange resin before analysis.

Calcium. Portions of hydrolysate (1 ml.) were diluted with 2 ml. of water and 1 ml. of 2·5 N-NaOH solution. The calcium was then titrated with 0·01 N-ethylenediaminetetra-acetic acid with the calcium indicator of Diehl & Ellingboe (1956), screened with thymolphthalein (Tucker, 1957).

Uronic acid. This was determined in 1 ml. portions of the hydrolysate by the method of Dische (1947). Glucuronolactone (L. Light and Co. Ltd.) was used for standardizing.

All chemical estimations were carried out in duplicate. No differences were found in the results from the male and female rabbits in each group.

RESULTS

The mean percentage composition of the ear cartilage is shown in Table 1, together with an analysis of variance of the individual results obtained in the 16 animals. It will be seen that papain significantly lowered the hexosamine and uronic acid content of the ear cartilage, irrespective of whether calciferol was administered simultaneously or not. In addition, calciferol resulted in a significant decrease in hexose, irrespective of whether papain was administered simultaneously. There was also an increase in calcium content of ear cartilage in the calciferol-treated rabbits, but the difference did not reach significant levels. Finally, the analysis shows that there was no significant interaction between papain and calciferol, i.e. they neither potentiated nor inhibited each other.

The weight of the fat-free aorta is expressed as dry wt./kg. body wt. As will be seen in Table 2, this weight was almost doubled in the calciferol-treated rabbits. Consequently, if the aortic constituents are expressed in terms of concentration, changes in their absolute values would be obscured. These values are therefore expressed as mg./aorta for a standard body weight (kg. body wt.).

Table 2 shows that calciferol resulted in significant increases in hexosamine and uronic acid (to just less than double) and an approximately 25-fold increase in the calcium content. These changes, induced by calciferol, were so marked that they tended, in the analysis of variance, to obscure changes induced by papain alone without simultaneous calciferol administration. A subsidiary analysis of the original data from groups I and II showed that papain significantly lowered the hexose, hexosamine and uronic acid content [Mann–Whitney *U* test (Siegal, 1956), $P < 0{\cdot}05$].

Because of the marked differences in standard error (and hence variance) of the calcium results in Table 2, doubt may be cast on the applicability of an analysis of variance in this case. The original data for each group were therefore subjected to a non-parametric test. Similar conclusions were

Table 1. *Chemical composition of rabbit-ear cartilage*

NS, Not significant.

	Percentage of dry, fat-free tissue (mean ± S.E.*)			
	Hexose	Hexosamine	Uronic acid	Calcium
Papain	2·75 ± 0·37	1·64 ± 0·15	1·45 ± 0·13	0·39 ± 0·03
0·9 % Sodium chloride	3·06 ± 0·04	1·99 ± 0·08	1·80 ± 0·08	0·42 ± 0·04
Papain + calciferol	2·15 ± 0·03	1·46 ± 0·19	1·43 ± 0·13	0·53 ± 0·11
Saline + calciferol	2·22 ± 0·18	2·04 ± 0·13	1·80 ± 0·16	0·63 ± 0·12

	Analysis of variance							
	F†	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main effects								
Papain	0·86	NS	8·72	<0·025	7·30	<0·025	0·56	NS
Calciferol	15·00	<0·01	0·10	NS	0·04	NS	4·52	>0·05
Interaction								
Papain + calciferol	0·29	NS	0·62	NS	0·04	NS	0·15	NS

* Standard error calculated as S.D./$\sqrt{(n-1)}$.
† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

Table 2. *Chemical composition of rabbit aorta*

NS, Not significant.

	Aortic wt. (mg./kg. body wt.)	Weights [mg./aorta/kg. body wt. (mean ± S.E.*)]			
		Hexose	Hexosamine	Uronic acid	Calcium
Papain	30·2 ± 3·8	0·30 ± 0·02	0·15 ± 0·01	0·11 ± 0·01	0·29 ± 0·09
0·9 % Sodium chloride	37·3 ± 3·9	0·41 ± 0·04	0·20 ± 0·02	0·19 ± 0·02	0·20 ± 0·04
Papain + calciferol	70·2 ± 9·7	0·46 ± 0·08	0·32 ± 0·04	0·25 ± 0·04	5·94 ± 1·05
0·9 % Sodium chloride + calciferol	65·2 ± 4·6	0·44 ± 0·04	0·32 ± 0·02	0·25 ± 0·02	6·48 ± 1·53

	Analysis of variance									
	F†	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main effects										
Papain	0·01	NS	0·98	NS	0·91	NS	2·60	NS	0·06	NS
Calciferol	29·41	<0·001	4·03	NS	31·52	<0·001	15·65	<0·01	41·23	<0·001
Interaction										
Papain + calciferol	0·97	NS	2·05	NS	1·30	NS	2·02	NS	0·12	NS

* Standard error calculated as S.D./$\sqrt{(n-1)}$.
† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

reached, namely that papain had no significant effect on aortic calcification whereas the differences induced by calciferol were highly significant (Mann–Whitney *U* test: $P < 0{\cdot}001$).

DISCUSSION

The present findings in ear cartilage are consistent with previous reports (Thomas, 1956; Tsaltas, 1958; Spicer & Bryant, 1958) that papain decreases the mucopolysaccharide content of rabbit-ear cartilage. It should be noted that papain induced a similar decrease in the hexose, hexosamine and uronic acid contents of the rabbit aorta.

On the other hand, the effects of calciferol were different in the two tissues analysed here. Whereas calciferol induced a massive mineralization of the aorta, the calcium content of the ear cartilage was only slightly increased. Calciferol decreased the hexose in ear cartilage and did not affect hexosamine and uronic acid contents, whereas in the aorta it resulted in an increase in both hexosamine and uronic acid, unassociated with any significant change in aortic hexose.

In the rat, calciferol administration results in increases in aortic hexose and hexosamine (Gillman *et al.* 1960) whereas the present results show no significant change in the hexose content of the calciferol-treated rabbit aorta. These two experiments were performed under different conditions, however, and a species difference cannot therefore be inferred from our data.

We wish to draw attention to the remarkable difference in sensitivity of the rabbit and the rat to calciferol: on a body-weight basis rats tolerated a

dose approximately 12 times as great as that tolerated by the rabbit.

In conclusion, from these results it would appear that although papain decreases the hexosamine and uronic acid content of the rabbit aorta, it has no effect on the degree of mineralization induced by calciferol administered simultaneously; nor does papain interfere with the ability of calciferol to raise the content of these mucopolysaccharides in the calcifying aorta.

SUMMARY

1. Chemical changes induced by papain and calciferol in rabbit-ear cartilage and aorta have been determined.

2. Papain significantly lowered the hexosamine and uronic acid contents of ear cartilage whether calciferol was given simultaneously or not. Calciferol lowered the hexose content when it was given alone or with papain.

3. The weight of dry aortic tissue was almost doubled in the calciferol-treated rabbits owing to heavy mineralization. Aortic hexosamine, uronic acid and calcium were increased in the calciferol-treated animals, whereas the hexose content was unchanged.

4. In the papain-treated animals there was a decrease in aortic hexosamine and uronic acid. However, when given simultaneously with calciferol, papain had no effect on the extent of mineralization.

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